CONTROL OF GENE EXPRESSION

This application is a continuation-in-part of U.S. Application No. 09/100,812.

FIELD OF THE INVENTION

The present invention relates generally to a method of modifying gene expression and to synthetic genes for modifying endogenous gene expression in a cell, tissue or organ of a transgenic organism, in particular a transgenic animal or plant. More particularly, the present invention utilises recombinant DNA technology to post-transcriptionally modify or modulate the expression of a target gene in a cell, tissue, organ or whole organism, thereby producing novel phenotypes. Novel synthetic genes and genetic constructs which are capable of repressing delaying or otherwise reducing the expression of an endogenous gene or a target gene in an organism when introduced thereto are also provided.

GENERAL

Bibliographic details of the publications referred to in this specification are collected at the end of the description.

As used herein the term "derived from" shall be taken to indicate that a specified

integer may be obtained from a particular specified source or species, albeit not necessarily directly from that specified source or species.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated step or element or integer or group of steps or elements or integers but not the exclusion of any other step or element or integer or group of elements or integers.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purposes of exemplification only. Functionally-equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

Sequence identity numbers (SEQ ID NOS.) containing nucleotide and amino acid sequence information included in this specification are collected after the Abstract and have been prepared using the programme PatentIn Version 2.0. Each nucleotide or amino acid sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (e.g. <210>1, <210>2, etc). The length, type of sequence (DNA, protein (PRT), etc) and source organism for each nucleotide or amino acid sequence are indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Nucleotide and amino acid sequences referred to in the specification are defined by the information provided in numeric indicator field <400> followed by the sequence identifier (eg. <400>1, <400>2, etc).

The designation of nucleotide residues referred to herein are those recommended by the IUPAC-IUB Biochemical Nomenclature Commission, wherein A represents Adenine, C represents Cytosine, G represents Guanine, T represents thymine, Y represents a pyrimidine residue, R represents a purine residue, M represents Adenine or Cytosine, K represents Guanine or Thymine, S represents Guanine or Cytosine, W represents Adenine or Thymine, H represents a nucleotide other than Guanine, B represents a nucleotide other than Adenine, V represents a nucleotide other than Thymine, D represents a nucleotide other than Cytosine and N represents any nucleotide residue.

The designation of amino acid residues referred to herein, as recommended by the IUPAC-IUB Biochemical Nomenclature Commission, are listed in Table 1.

TABLE 1

Amino Acid	Three-letter code	One-letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Aspartate/Asparagine	Baa	B
Glutamate/Glutamine	Zaa	Z
Any amino acid	Xaa	X

BACKGROUND TO THE INVENTION

Controlling metabolic pathways in eukaryotic organisms is desirable for the purposes of producing novel traits therein or introducing novel traits into a particular cell, tissue or organ of said organism. Whilst recombinant DNA technology has provided significant progress in an understanding of the mechanisms regulating eukaryotic gene expression, much less progress has been made in the actual manipulation of gene expression to produce novel traits. Moreover, there are only limited means by which human intervention may lead to a modulation of the level of eukaryotic gene expression.

One approach to repressing, delaying or otherwise reducing gene expression utilises an RNA molecule which is transcribed from the complementary strand of a nuclear gene to that which is normally transcribed and capable of being translated into a polypeptide. Although the precise mechanism involved in this approach is not established, it has been postulated that a double-stranded RNA may form by base pairing between the complementary nucleotide sequences, to produce a complex which is translated at low efficiency and/or degraded by intracellular ribonuclease enzymes prior to being translated.

Alternatively, the expression of an endogenous gene in a plant cell, tissue or organ may be suppressed when one or more copies of said gene, or one or more copies of a substantially similar gene are introduced into the cell. Whilst the mechanism involved in this phenomenon has not been established it appears to involve mechanistically heterogeneous processes. For example, this approach has been postulated to involve transcriptional repression, in which case somatically-heritable repressed states of chromatin are formed or alternatively, a post-transcriptional silencing wherein transcription occurs normally but the RNA products of the co-suppressed genes are subsequently eliminated.

The efficiency of both of these approaches in targeting the expression of specific genes is very low and highly variable results are usually obtained. Inconsistent results are obtained using different regions of genes, for example 5´- untranslated

regions, 3´-untranslated regions, coding regions or intron sequences to target gene expression. Accordingly, there currently exists no consensus as to the nature of genetic sequences which provide the most efficient means for repressing, delaying or otherwise reducing gene expression using existing technologies. Moreover, such a high degree of variation exists between generations that it is not possible to predict the level of repression of a specific gene in the progeny of an organism in which gene expression was markedly modified.

Recently, Dorer and Henikoff (1994) demonstrated the silencing of tandemly repeated gene copies in the *Drosophila* genome and the transcriptional repression of dispersed *Drosophila Adh* genes by *Polycomb* genes (i.e. the *Pc-G* system; Pal-Bhadra *et al*, 1997). However, such silencing of tandemly repeated gene copies is of little utility in an attempt to manipulate gene expression in an animal cell by recombinant means, wherein the sequences capable of targeting the expression of a particular gene are introduced at dispersed locations in the genome, absent the combination of this approach with gene-targeting technology. Whilst theoretically possible, such combinations would be expected to work at only low-efficiency, based upon the low efficiency of gene-targeting approaches used in isolation and further, would require complicated vector systems. Additionally, the utilisation of transcriptional repression, such as the *Drosophila Pc-G* system, would appear to require some knowledge of the regulatory mechanisms capable of modulating the expression of any specific target gene and, as a consequence, would be difficult to implement in practice as a general technology for repressing, delaying or reducing gene expression in animal cells.

The poor understanding of the mechanisms involved in these phenomena has meant that there have been few improvements in technologies for modulating the level of gene expression , in particular technologies for delaying, repressing or otherwise reducing the expression of specific genes using recombinant DNA technology. Furthermore, as a consequence of the unpredictability of these approaches, there is currently no commercially-viable means for modulating the level of expression of a specific gene in a eukaryotic or prokaryotic organism.

Thus, there exists a need for improved methods of modulating gene expression, in particular repressing, delaying or otherwise reducing gene expression in animal cells for the purpose of introducing novel phenotypic traits thereto. In particular, these methods should provide general means for phenotypic modification, without the necessity for performing concomitant gene-targeting approaches.

SUMMARY OF THE INVENTION

The invention is based in part on the surprising discovery by the inventors that cells which exhibit one or more desired traits can be produced and selected from transformed cells comprising a nucleic acid molecule operably linked to a promoter, wherein the transcription product of the nucleic acid molecule comprises a nucleotide sequence which is substantially identical to the nucleotide sequence of a transcript of an endogenous or non-endogenous target gene, the expression of which is intended to be modulated. The transformed cells are regenerated into whole tissues, organs or organisms capable of exhibiting novel traits, in particular virus resistance and modified expression of endogenous genes.

Accordingly, one aspect of the present invention provides a method of modulating the expression of a target gene in a plant cell, tissue or organ comprising (a) providing one or more dispersed or foreign nucleic acid molecules which include multiple copies of a nucleotide sequence, each of which is substantially identical to or complementary to the nucleotide sequence of the target gene or a region thereof, and (b) transfecting the plant cell, tissue or organ with the dispersed or foreign nucleic acid molecules for a time and under conditions sufficient for expression of at least two of the multiple copies.

In a particularly preferred embodiment, either (a) at least two of the copies are in tandem and the same orientation, or (b) at least one of the copies is in the sense orientation and one is in the antisense orientation and these two copies are located relative to each other such that the two copies may form a hairpin RNA structure when transcribed.

The target gene may be a gene which is endogenous to the cell or alternatively, a foreign gene such as a viral or foreign genetic sequence, amongst others. Preferably, the target gene is a viral genetic sequence.

The invention is particularly useful in the modulation of eukaryotic gene expression, in particular the modulation of plant or animal gene expression and even more particularly in the modulation of expression of genes derived from crops, vertebrate and invertebrate animals, such as insects, aquatic animals (eg. fish, shellfish, molluscs, crustaceans such as crabs, lobsters and prawns, avian animals and mammals, amongst others).

A variety of traits are selectable with appropriate procedures and sufficient numbers of transformed cells. Such traits include, but are not limited to, visible traits, disease-resistance traits, and pathogen-resistance traits. The modulatory effect is applicable to a variety of genes expressed in animals including, for example, endogenous genes responsible for cellular metabolism or cellular transformation, including oncogenes, transcription factors and other genes which encode polypeptides involved in cellular metabolism.

For example, an alteration in the pigment production in mice can be engineered by targeting the expression of the tyrosinase gene therein. This provides a novel phenotype of albinism in black mice. By targeting genes required for virus replication in a plant cell or an animal cell, a genetic construct which comprises multiple copies of nucleotide sequence encoding a viral replicase, polymerase, coat protein or uncoating gene, or protease protein, may be introduced into a cell where it is expressed, to confer immunity against the virus upon the cell.

In performance of the present invention, the dispersed nucleic acid molecule or foreign nucleic acid molecule will generally comprise a nucleotide sequence having greater than about 85% identity to the target gene sequence, however, a higher homology might produce a more effective modulation of expression of the target gene sequence. Substantially greater homology, or more than about 90% is preferred, and even more preferably about 95% to absolute identity is desirable.

The introduced dispersed nucleic acid molecule or foreign nucleic acid molecule sequence, needing less than absolute homology, also need not be full length, relative to either the primary transcription product or fully processed mRNA of the target gene. A higher homology in a shorter than full length sequence compensates for a longer less homologous sequence. Furthermore, the introduced sequence need not have the same intron or exon pattern, and homology of non-coding segments will be equally effective.

A second aspect of the present invention provides a method of modulating the expression of a target gene in a plant cell, tissue or organ, said method comprising:

(i) selecting one or more dispersed nucleic acid molecules or foreign nucleic acid molecules which comprise multiple copies of a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a region thereof or which is complementary thereto;

(ii) producing a synthetic gene comprising said dispersed nucleic acid molecules or foreign nucleic acid molecules operably connected to a promoter sequence operable in said cell, tissue or organ;

(iii) introducing said synthetic gene to said cell, tissue or organ; and

(iv) expressing said synthetic gene in said cell, tissue or organ for a time and under conditions sufficient for expression of at least two of the copies.

A third aspect of the invention provides a method of conferring resistance or immunity to a viral pathogen upon a plant cell, tissue, organ or whole organism, comprising:

(i) selecting one or more dispersed nucleic acid molecules or foreign nucleic acid molecules which comprise multiple copies substantially identical to a nucleotide sequence derived from the viral pathogen or a complementary sequence thereto or a region thereof;

(ii) producing a synthetic gene comprising said dispersed nucleic acid molecules or foreign nucleic acid molecules operably connected to a promoter sequence operable in said cell, tissue, organ or whole organism;

(iii) introducing said synthetic gene to said cell, tissue, organ or whole organism; and

(iv) expressing said synthetic gene in said cell, tissue or organ for a time and under conditions sufficient for expression of at least two of the copies.

A fourth aspect of the present invention provides a genetic construct comprising multiple structural gene sequences, wherein each of said structural gene sequences is substantially identical to a target gene in a plant cell, and wherein said multiple structural gene sequences are placed operably under the control of a single promoter sequence which is operable in said cell, wherein at least one of said structural gene sequences is placed operably in the sense orientation under the control of said promoter sequence and at least one other of said structural gene sequences is placed operably in the antisense orientation under the control of said promoter sequence, and wherein at least one structural gene sequence that is placed in the sense orientation relative to said promoter and at least one structural gene sequence that is placed in the antisense orientation relative to said promoter are spaced from each other by a nucleic acid stuffer fragment.

A fifth aspect of the present invention provides a genetic construct which is capable of modulating the expression of a target gene in a plant cell, which is transfected with said construct, wherein said construct comprises multiple copies

of a structural gene sequence, wherein each copy comprises a nucleotide sequence which is substantially identical to said target gene or a derivative of said target gene and wherein said multiple copies are placed operably under the control of a single promoter sequence which is operable in said cell, wherein at least two of said copies are placed operably in the sense orientation under the control of said promoter sequence.

A sixth aspect of the present invention provides a genetic construct which is capable of modulating the expression of a target gene in a plant cell, which is transfected with said construct, wherein said construct comprises multiple structural gene sequences wherein each of said structural gene sequences is separately placed under the control of a promoter which is operable in said cell, and wherein each of said structural gene sequences comprises a nucleotide sequence which is substantially identical to said target gene or a derivative of said target gene, wherein at least one of said structural gene sequences is placed operably in the sense orientation under the control of an individual promoter sequence.

In order to observe many novel traits in multicellular organisms in particular those which are tissue-specific or organ-specific or developmentally-regulated, regeneration of a transformed cell carrying the synthetic genes and genetic constructs described herein into a whole organism will be required. Those skilled in the art will be aware that this means growing a whole organism from a transformed animal cell, a group of such cells, a tissue or organ. Standard methods for the regeneration of certain animals from isolated cells and tissues are known to those skilled in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagrammatic representation of the plasmid pEGFP-N1 MCS.

Figure 2 is a diagrammatic representation of the plasmid pCMV.cass.

Figure 3 is a diagrammatic representation of the plasmid pCMV.SV40L.cass.

Figure 4 is a diagrammatic representation of the plasmid pCMV.SV40LR.cass.

Figure 5 is a diagrammatic representation of the plasmid pCR.Bgl-GFP-Bam.

Figure 6 is a diagrammatic representation of the plasmid pBSII(SK+).EGFP.

Figure 7 is a diagrammatic representation of the plasmid pCMV.EGFP.

Figure 8 is a diagrammatic representation of the plasmid pCR.SV40L.

Figure 9 is a diagrammatic representation of the plasmid pCR.BEV.1.

Figure 10 is a diagrammatic representation of the plasmid pCR.BEV.2.

Figure 11 is a diagrammatic representation of the plasmid pCR.BEV.3.

Figure 12 is a diagrammatic representation of the plasmid pCMV.EGFP.BEV2.

Figure 13 is a diagrammatic representation of the plasmid pCMV.BEV.2.

Figure 14 is a diagrammatic representation of the plasmid pCMV.BEV.3.

Figure 15 is a diagrammatic representation of the plasmid pCMV.VEB.

Figure 16 is a diagrammatic representation of the plasmid pCMV.BEV.GFP.

Figure 17 is a diagrammatic representation of the plasmid pCMV.BEV.SV40L-0.

Figure 18 is a diagrammatic representation of the plasmid pCMV.0.SV40L.BEV.

Figure 19 is a diagrammatic representation of the plasmid pCMV.0.SV40L.VEB.

Figure 20 is a diagrammatic representation of the plasmid pCMV.BEVx2.

Figure 21 is a diagrammatic representation of the plasmid pCMV.BEVx3.

Figure 22 is a diagrammatic representation of the plasmid pCMV.BEVx4.

Figure 23 is a diagrammatic representation of the plasmid pCMV.BEV.SV40L.BEV.

Figure 24 is a diagrammatic representation of the plasmid pCMV.BEV.SV40L.VEB.

Figure 25 is a diagrammatic representation of the plasmid pCMV.BEV.GFP.VEB.

Figure 26 is a diagrammatic representation of the plasmid pCMV.EGFP.BEV2.PFG.

Figure 27 is a diagrammatic representation of the plasmid pCMV.BEV.SV40LR.

Figure 28 is a diagrammatic representation of the plasmid pCDNA3.Galt.

Figure 29 is a diagrammatic representation of the plasmid pCMV.Galt.

Figure 30 is a diagrammatic representation of the plasmid pCMV.EGFP.Galt.

Figure 31 is a diagrammatic representation of the plasmid pCMV.Galt.GFP.

Figure 32 is a diagrammatic representation of the plasmid pCMV.Galt.SV40L.0.

Figure 33 is a diagrammatic representation of the plasmid pCMV.Galt.SV40L.tlaG.

Figure 34 is a diagrammatic representation of the plasmid pCMV.0.SV40L.Galt.

Figure 35 is a diagrammatic representation of the plasmid pCMV.Galtx2.

Figure 36 is a diagrammatic representation of the plasmid pCMV.Galtx4.

Figure 37 is a diagrammatic representation of the plasmid pCMV.Galt.SV40L.Galt.

Figure 38 is a diagrammatic representation of the plasmid pCMV.Galt.SV40L.tlaG.

Figure 39 is a diagrammatic representation of the plasmid pCMV.Galt.GFP.tlaG.

Figure 40 is a diagrammatic representation of the plasmid pCMV.EGFP.Galt.PFG.

Figure 41 is a diagrammatic representation of the plasmid pCMV.Galt.SV40LR.

Figure 42 is a diagrammatic representation of the plasmid pART7.

Figure 43 is a diagrammatic representation of the plasmid pART7.35S.SCBV.cass.

Figure 44 is a diagrammatic representation of the plasmid pBC.PVY.

Figure 45 is a diagrammatic representation of the plasmid pSP72.PVY.

Figure 46 is a diagrammatic representation of the plasmid pClapBC.PVY.

Figure 47 is a diagrammatic representation of the plasmid pBC.PVYx2.

Figure 48 is a diagrammatic representation of the plasmid pSP72.PVYx2.

Figure 49 is a diagrammatic representation of the plasmid pBC.PVYx3.

Figure 50 is a diagrammatic representation of the plasmid pBC.PVYx4.

Figure 51 is a diagrammatic representation of the plasmid pBC.PVY.LNYV.

Figure 52 is a diagrammatic representation of the plasmid pBC.PVY.LNYV.PVY.

Figure 53 is a diagrammatic representation of the plasmid pBC.PVY.LNYV.YVP• .

Figure 54 is a diagrammatic representation of the plasmid pBC.PVY.LNYV.YVP.

Figure 55 is a diagrammatic representation of the plasmid pART27.PVY

Figure 56 is a diagrammatic representation of the plasmid pART27.35S.PVY.SCBV.O.

Figure 57 is a diagrammatic representation of the plasmid pART27.35S.O.SCBV.PVY.

Figure 58 is a diagrammatic representation of the plasmid pART27.35S.O.SCBV.YVP.

Figure 59 is a diagrammatic representation of the plasmid pART7.PVYx2.

Figure 60 is a diagrammatic representation of the plasmid pART7.PVYx3.

Figure 61 is a diagrammatic representation of the plasmid pART7.PVYx4.

Figure 62 is a diagrammatic representation of the plasmid pART7.PVY.LNYV.PVY.

Figure 63 is a diagrammatic representation of the plasmid pART7.PVY.LNYV.YVP.

Figure 64 is a diagrammatic representation of the plasmid pART7.PVY.LNYV.YVP.

Figure 65 is a diagrammatic representation of pART7.35S.PVY.SCBV.YVP.

Figure 66 is a diagrammatic representation of pART7.35S.PVYx3.SCBV.YVPx3.

Figure 67 is a diagrammatic representation of pART7.PVYx3.LNYV.YVPx3.

Figure 68 is a diagrammatic representation of the plasmid pART7.PVYMULTI.

Figure 69 shows micrographs of PK-1 cell lines transformed with pCMV.EGFP, viewed under normal light and under fluorescence conditions (excitation λ = 488 nm, emission λ = 507 nm) designed to detect GFP. A: PK EGFP 2.11 cells under normal light; B: PK EGFP 2.11 cells under fluorescence conditions; C: PK EGFP 2.18 cells under normal light; D: PK EGFP 2.18 cells under fluorescence conditions.

Figure 70 shows an example of Southern blot analysis of transgenic porcine kidney cells (PK) which had been transformed with the construct pCMV.EGFP. Genomic DNA was isolated from PK-1 cells and transformed lines, digested with the restriction endonuclease *Bam*H1 and probed with a ^{32}P-dCTP labeled EGFP DNA fragment. Lane A is a molecular weight marker where sizes of each fragment are indicated in kilobases (kb); Lane B is the parental cell line PK-1. Lane C is A4, a transgenic EGFP-expressing PK-1 cell line; Lane D is C9, a transgenic non-expressing PK-1 cell line.

Figure 71 shows micrographs of CRIB-1 cells and a CRIB-1 transformed line [CRIB-1 BGI2 # 19(tol)] prior to and 48 hrs after infection with identical titres of BEV. A: CRIB-1 cells prior to BEV infection; B: CRIB-1 cells 48 hrs after BEV infection; C: CRIB-1 BGI2 # 19(tol) cells prior to infection with BEV; D: CRIB-1 BGI2 # 19(tol) 48 hrs after BEV infection. For further details refer to Example 8.

Figure 72 shows levels of pigmentation in B16 cells and B16 cells transformed with pCMV.TYR.BGI2.RYT. Cell lines are, from left to right: B16, B16 2.1.6, B16 2.1.11, B16 3.1.4, B16 3.1.15, B16 4.12.2 and B16 4.12.3. For further details refer to Example 9.

Figure 73 shows immunofluorescent micrographs of MDA-MB-468 cells and MDA-MB-468 cells transformed with pCMV.HER2.BGI2.2REH stained for HER-2. A: MDA-MB-468 cells; B: MDA-MB-468 cells stained with only the secondary antibody; C: MDA-MB-468 1.4 cells stained for HER-2; D: MDA-MB-468 1.10 cells

stained for HER-2. For further details refer to Example 10.

Figure 74 shows FACS analyses of HER-2 expression in (A) MDA-MB-468 cells; (B) MDA-MB-468 1.4 cells; (C) MDA-MB-468 1.10 cells. For further details refer to Example 10.

Figure 75 is a histograph showing viable cell counts after transfection with YB-1-related gene constructs and oligonucleotides. Viable cells were counted in quadruplicate samples with a haemocytometer following staining with trypan blue. Column heights show the average cell count of two independent transfection experiments and vertical bars indicate the standard deviation. (A) Viable B10.2 cell counts 72 hr after transfection with gene constructs: (i) control: pCMV.EGFP; (ii) pCMV.YB1.BGI2.1BY; (iii) pCMV.YB1.p53.BGI2.35p.1BY. All materials and procedures used are described in the text for Example 11. (B) Viable Pam 212 cell counts 72 hr after transfection with gene constructs: (i) control: pCMV.EGFP; (ii) pCMV.YB1.BGI2.1BY; (iii) pCMV.YB1.p53.BGI2.35p.1BY. All materials and procedures used are described in the text for Example 11. (C) Viable B10.2 cell counts 18 hr after transfection with oligonucleotides: (i) control: Lipofectin (trademark) only; (ii) control: non-specific oligonucleotide; (iii) decoy Y-box oligonucleotide. All materials and procedures used are described in the text for Example 11. (D) Viable Pam 212 cell counts 18 hr after transfection with oligonucleotides: (i) control: Lipofectin (trademark) only; (ii) control: non-specific oligonucleotide; (iii) decoy Y-box oligonucleotide. All materials and procedures used are described in the text for Example 11.

Figure 76: A Northern blot showing levels of GFP expression in MM96L cells and MM96L lines transformed with pCMV.EGFP. 10 μg of total RNA from the indicated cell lines were electrophoresed on agarose gels and transferred to a nylon membrane. The filter was probed with a radio-labelled fragment derived from the EGFP gene. **B** Photograph showing ethidium bromide-stained ribosomal RNAs from the RNA samples probed in A; the equal intensities indicated similar amounts of RNA from each cell line were probed.

Figure 77: Real-Time RT-PCR analysis of transformed cell lines for EGFP mRNA levels and EGFP RNA transcribed from the EGFP transgene in nuclear run-on assays. **A** EGFP mRNA levels in MM96L lines 3, 9 and 18 (as in Figure 76). **B** EGFP gene run-on transcripts in nuclei of MM96L lines 3, 9 and 18 (as in Figure 76). **C** Glyceraldehyde phosphate dehydrogenase (GAPD) mRNA levels in MM96L lines 3, 9 and 18(as in Figure 76). **D** GAPD gene run-on transcripts in nuclei of MM96L lines 3, 9 and 18 (as in Figure 76).

Figure 78: Relative mRNA levels and RNA transcribed from the EGFP transgene in nuclear run-on assays, from the data shown in Figure 77. The EGFP gene in line 9 is transcribed but EGFP mRNA levels are extremely low, signifying post-transcriptional gene silencing (co-suppression).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of modulating the expression of a target gene in a cell, tissue or organ, said method at least comprising the step of introducing to said cell, tissue or organ one or more dispersed nucleic acid molecules or foreign nucleic acid molecules comprising multiple copies of a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a region thereof or complementary thereto for a time and under conditions sufficient for translation of the mRNA product of said target gene to be modified, subject to the proviso that the transcription of said mRNA product is not exclusively repressed or reduced.

By "multiple copies" is meant that two or more substantially identical (as defined below) copies of a nucleotide sequence are present in the same or different orientation, on the same nucleic acid molecule. As would be appreciated by one skilled in the art, the term "direct repeat" is used in contradistinction to the term "inverted repeat" such that a direct repeat is a 5'-3'.5'-3' repeat (with or without other nucleotides between the repeated sequences). An inverted repeat is a 5'-3'.3'-5' sequence (the 3'-5' sequence may also be called a "reverse complement" or antisense of the 5'-3' sequence) such that the transcription product of the

inverted repeat (e.g. mRNA) may form a hairpin RNA structure. Further, the term "tandem" is used by those skilled in the art to indicate repeats separated by no or relatively few (relative to the length of the repeated sequence) intervening nucleotides.

Repeats may optionally be separated by a stuffer fragment or intergenic region to facilitate secondary structure formation between each repeat where this is required. The stuffer fragment may comprise any combination of nucleotide or amino acid residues, carbohydrate molecules or oligosaccharide molecules or carbon atoms or a homologue, analogue or derivative thereof which is capable of being linked covalently to a nucleic acid molecule.

Preferably, the stuffer fragment comprises a sequence of nucleotides or a homologue, analogue or derivative thereof.

Where the dispersed or foreign nucleic acid molecule includes intron/exon splice junction sequences, the stuffer fragment may serve as an intron sequence placed between the 3´-splice site of the structural gene nearer the 5´-end of the gene and the 5´- splice site of the next downstream unit thereof. Alternatively, where it is desirable for more than two adjacent nucleotide sequence units of the dispersed foreign nucleic acid molecule to be translated, the stuffer fragment placed there between should not include an in-frame translation stop codon, absent intron/exon splice junction sequences at both ends of the stuffer fragment or the addition of a translation start codon at the 5´ end of each unit, as will be obvious to those skilled in the art.

Stuffer fragments can include those which encode detectable marker proteins or biologically-active analogues and derivatives thereof, for example luciferase, β-galacturonase, β-galactosidase, chloramphenicol acetyltransferase or green fluorescent protein, amongst others. Additional stuffer fragments are not excluded.

According to this embodiment, the detectable marker or an analogue or derivative

thereof serves to indicate the expression of the synthetic gene of the invention in a cell, tissue or organ by virtue of its ability to confer a specific detectable phenotype thereon, preferably a visually-detectable phenotype.

As used herein, the term "modulating" shall be taken to mean that expression of the target gene is reduced in amplitude and/or the timing of gene expression is delayed and/or the developmental or tissue-specific or cell-specific pattern of target gene expression is altered, compared to the expression of said gene in the absence of the inventive method described herein.

Whilst not limiting the scope of the invention described herein, the present invention is directed to a modulation of gene expression which comprises the repression, delay or reduction in amplitude of target gene expression in a specified cell, tissue or organ of a eukaryotic organism, in particular a plant such as a monocotyledonous or dicotyledonous plant, or a human or other animal and even more particularly a vertebrate and invertebrate animal, such as an insect, aquatic animal (eg. fish, shellfish, mollusc, crustacean such as a crab, lobster or prawn, an avian animal or a mammal, amongst others).

More preferably, target gene expression is completely inactivated by the dispersed nucleic acid molecules or foreign nucleic acid molecules which has been introduced to the cell, tissue or organ.

Whilst not being bound by any theory or mode of action, the reduced or eliminated expression of the target gene which results from the performance of the invention may be attributed to reduced or delayed translation of the RNA transcription product of the target gene or alternatively, the prevention of translation of said RNA, as a consequence of sequence-specific degradation of the RNA transcript of the target gene by an endogenous host cell system.

It is particularly preferred that, for optimum results, sequence-specific degradation of the RNA transcript of the target gene occurs either prior to the time or stage when the RNA transcript of the target gene would normally be translated or

alternatively, at the same time as the RNA transcript of the target gene would normally be translated. Accordingly, the selection of an appropriate promoter sequence to regulate expression of the introduced dispersed nucleic acid molecule or foreign nucleic acid molecule is an important consideration to optimum performance of the invention. For this reason, strong constitutive promoters or inducible promoter systems are especially preferred for use in regulating expression of the introduced dispersed nucleic acid molecules or foreign nucleic acid molecules.

The present invention clearly encompasses reduced expression wherein reduced expression of the target gene is effected by lowered transcription, subject to the proviso that a reduction in transcription is not the sole mechanism by which this occurs and said reduction in transcription is at least accompanied by reduced translation of the steady-state mRNA pool of the target gene.

The target gene may be a genetic sequence which is endogenous to the cell or alternatively, a non-endogenous genetic sequence, such as a genetic sequence which is derived from a virus or other foreign pathogenic organism and is capable of entering a cell and using the cell's machinery following infection.

Where the target gene is a non-endogenous genetic sequence to the cell, it is desirable that the target gene encodes a function which is essential for replication or reproduction of the viral or other pathogen. In such embodiments, the present invention is particularly useful in the prophylactic and therapeutic treatment of viral infection of an animal cell or for conferring or stimulating resistance against said pathogen.

Preferably, the target gene comprises one or more nucleotide sequences of a viral pathogen of a plant or an animal cell, tissue or organ.

For example, in the case of animals and humans, the viral pathogen may be a retrovirus, for example a lentivirus such as the immunodeficiency viruses, a single-stranded (+) RNA virus such as bovine enterovirus (BEV) or Sinbis alphavirus.

Alternatively, the target gene can comprise one or more nucleotide sequences of a viral pathogen of an animal cell, tissue or organ, such as but not limited to a double-stranded DNA virus such as bovine herpes virus or herpes simplex virus I (HSV I), amongst others.

In the case of plants, the virus pathogen is preferably a potyvirus, caulimovirus, badnavirus, geminivirus, reovirus, rhabdovirus, bunyavirus, tospovirus, tenuivirus, tombusvirus, luteovirus, sobemovirus, bromovirus, cucomovirus, ilavirus, alfamovirus, tobamovirus, tobravirus, potexvirus and clostrovirus, such as but not limited to CaMV, SCSV, PVX, PVY, PLRV, and TMV, amongst others.

With particular regard to viral pathogens, those skilled in the art are aware that virus-encoded functions may be complemented *in trans* by polypeptides encoded by the host cell. For example, the replication of the bovine herpes virus genome in the host cell may be facilitated by host cell DNA polymerases which are capable of complementing an inactivated viral DNA polymerase gene.

Accordingly, where the target gene is a non-endogenous genetic sequence to the animal cell, a further alternative embodiment of the invention provides for the target gene to encode a viral or foreign polypeptide which is not capable of being complemented by a host cell function, such as a virus-specific genetic sequence. Exemplary target genes according to this embodiment of the invention include, but are not limited to genes which encode virus coat proteins, uncoating proteins and RNA-dependent DNA polymerases and RNA-dependent RNA polymerases, amongst others.

In a particularly preferred embodiment of the present invention, the target gene is the BEV RNA-dependent RNA polymerase gene or a homologue, analogue or derivative thereof or PVY Nia protease-encoding sequences.

The cell in which expression of the target gene is modified may be any cell which is derived from a multicellular animal, including cell and tissue cultures thereof. Preferably, the animal cell is derived from an anthropod, nematode, reptile,

amphibian, bird, human or other mammal. Exemplary animal cells include embryonic stem cells, cultured skin fibroblasts, neuronal cells, somatic cells, haematopoietic stem cells, T-cells and immortalised cell lines such as COS, VERO, HeLa, mouse C127, Chinese hamster ovary (CHO), WI-38, baby hamster kidney (BHK) or MDBK cell lines, amongst others. Such cells and cell lines are readily available to those skilled in the art. Accordingly, the tissue or organ in which expression of the target gene is modified may be any tissue or organ comprising such animal cells.

Preferably the plant cell is derived from a monocotyledonous or dicotyledonous plant species or a cell line derived therefrom.

As used herein, the term "dispersed nucleic acid molecule" shall be taken to refer to a nucleic acid molecule which comprises one or more multiple copies of a nucleotide sequence which is substantially identical or complementary to the nucleotide sequence of a gene which originates from the cell, tissue or organ into which said nucleic acid molecule is introduced, wherein said nucleic acid molecule is non-endogenous in the sense that it is introduced to the cell, tissue or organ of an animal via recombinant means and will generally be present as extrachromosomal nucleic acid or alternatively, as integrated chromosomal nucleic acid which is genetically-unlinked to said gene. More particularly, the "dispersed nucleic acid molecule" will comprise chromosomal or extrachromosomal nucleic acid which is unlinked to the target gene against which it is directed in a physical map, by virtue of their not being tandemly-linked or alternatively, occupying a different chromosomal position on the same chromosome or being localised on a different chromosome or present in the cell as an episome, plasmid, cosmid or virus particle.

By "foreign nucleic acid molecule" is meant an isolated nucleic acid molecule which has one or more multiple copies of a nucleotide sequence which originates from the genetic sequence of an organism which is different from the organism to which the foreign nucleic acid molecule is introduced. This definition encompasses a nucleic acid molecule which originates from a different individual of the same

lowest taxonomic grouping (i.e. the same population) as the taxonomic grouping to which said nucleic acid molecule is introduced, as well as a nucleic acid molecule which originates from a different individual of a different taxonomic grouping as the taxonomic grouping to which said nucleic acid molecule is introduced, such as a gene derived from a viral pathogen.

Accordingly, a target gene against which a foreign nucleic acid molecule acts in the performance of the invention may be a nucleic acid molecule which has been introduced from one organism to another organism using transformation or introgression technologies. Exemplary target genes according to this embodiment of the invention include the green fluorescent protein-encoding gene derived from the jellyfish *Aequoria victoria* (Prasher *et al.*,1992; International Patent Publication No. WO 95/07463), tyrosinase genes and in particular the murine tyrosinase gene (Kwon *et al.*,1988), the *Escherichia coli lacI* gene which is capable of encoding a polypeptide repressor of the *lacZ* gene, the porcine β-1,3-galactosyltransferase gene (NCBI Accession No. L36535) exemplified herein, and the PVY and BEV structural genes exemplified herein or a homologue, analogue or derivative of said genes or a complementary nucleotide sequence thereto.

The present invention is further useful for simultaneously targeting the expression of several target genes which are co-expressed in a particular cell, for example by using a dispersed nucleic acid molecule or foreign nucleic acid molecule which comprises nucleotide sequences which are substantially identical to each of said co-expressed target genes.

By "substantially identical" is meant that the introduced dispersed or foreign nucleic acid molecule of the invention and the target gene sequence are sufficiently identical at the nucleotide sequence level to permit base-pairing there between under standard intracellular conditions.

Preferably, the nucleotide sequence of each repeat in the dispersed or foreign nucleic acid molecule of the invention and the nucleotide sequence of a part of the target gene sequence are at least about 80-85% identical at the nucleotide

sequence level, more preferably at least about 85-90% identical, even more preferably at least about 90-95% identical and still even more preferably at least about 95-99% or 100% identical at the nucleotide sequence level.

Notwithstanding that the present invention is not limited by the precise number of repeated sequences in the dispersed nucleic acid molecule or the foreign nucleic acid molecule of the invention, it is to be understood that the present invention requires at least two copies of the dispersed foreign nucleic acid molecule of the target gene sequence to be expressed in the cell.

Preferably, the multiple copies of the target gene sequence are presented in the dispersed nucleic acid molecule or the foreign nucleic acid molecule as tandem inverted repeat sequences and/or tandem direct repeat sequences. Such configurations are exemplified by the "test plasmids" described herein that comprise Galt, BEV or PVY gene regions.

Preferably, the dispersed or foreign nucleic acid molecule which is introduced to the cell, tissue or organ comprises RNA or DNA.

Preferably, the dispersed or foreign nucleic acid molecule further comprises a nucleotide sequence or is complementary to a nucleotide sequence which is capable of encoding an amino acid sequence encoded by the target gene.

Standard methods may be used to introduce the dispersed nucleic acid molecule or foreign nucleic acid molecule into the cell, tissue or organ for the purposes of modulating the expression of the target gene. For example, the nucleic acid molecule may be introduced as naked DNA or RNA, optionally encapsulated in a liposome, in a virus particle as attenuated virus or associated with a virus coat or a transport protein or inert carrier such as gold or as a recombinant viral vector or bacterial vector or as a genetic construct, amongst others.

Administration means include injection and oral ingestion (e.g. in medicated food material), amongst others.

The subject nucleic acid molecules may also be delivered by a live delivery system such as using a bacterial expression system optimised for their expression in bacteria which can be incorporated into gut flora. Alternatively, a viral expression system can be employed. In this regard, one form of viral expression is the administration of a live vector generally by spray, feed or water where an infecting effective amount of the live vector (e.g. virus or bacterium) is provided to an animal. Another form of viral expression system is a non-replicating virus vector which is capable of infecting a cell but not replicating therein. The non-replicating viral vector provides a means of introducing to the human or animal subject genetic material for transient expression therein. The mode of administering such a vector is the same as a live viral vector.

The carriers, excipients and/or diluents utilised in delivering the subject nucleic acid molecules to a host cell should be acceptable for human or veterinary applications. Such carriers, excipients and/or diluents are well-known to those skilled in the art. Carriers and/or diluents suitable for veterinary use include any and all solvents, dispersion media, aqueous solutions, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the composition is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

In an alternative embodiment, the invention provides a method of modulating the expression of a target gene in a cell, tissue or organ, said method at least comprising the steps of:

(i) selecting one or more dispersed nucleic acid molecules or foreign nucleic acid molecules which comprise multiple copies of a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a region thereof or which is complementary thereto; and

(ii) introducing said dispersed nucleic acid molecules or foreign nucleic acid molecules to said cell, tissue or organ for a time and under conditions

sufficient for translation of the RNA product of said target gene to be modified, subject to the proviso that the transcription of said RNA product is not exclusively repressed or reduced.

To select appropriate nucleotide sequences for targeting expression of the target gene, several approaches may be employed. In one embodiment, multiple copies of specific regions of characterised genes may be cloned in operable connection with a suitable promoter and assayed for efficacy in reducing target gene expression. Alternatively, shotgun libraries comprising multiple copies of genetic sequences may be produced and assayed for their efficacy in reducing target gene expression. The advantage associated with the latter approach is that it is not necessary to have any prior knowledge of the significance of any particular target gene in specifying an undesirable phenotype in the cell. For example, shotgun libraries comprising virus sub-genomic fragments may be employed and tested directly for their ability to confer virus immunity on the animal host cell, without prior knowledge of the role which any virus genes play in pathogenesis of the host cell.

As used herein, the term "shotgun library" is a set of diverse nucleotide sequences wherein each member of said set is preferably contained within a suitable plasmid, cosmid, bacteriophage or virus vector molecule which is suitable for maintenance and/or replication in a cellular host. The term "shotgun library" includes a representative library, in which the extent of diversity between the nucleotide sequences is numerous such that all sequences in the genome of the organism from which said nucleotide sequences is derived are present in the "set" or alternatively, a limited library in which there is a lesser degree of diversity between said sequences. The term "shotgun library" further encompasses random nucleotide sequences, wherein the nucleotide sequence comprises viral or cellular genome fragments, amongst others obtained for example by shearing or partial digestion of genomic DNA using restriction endonucleases, amongst other approaches. A "shotgun library" further includes cells, virus particles and bacteriophage particles comprising the individual nucleotide sequences of the diverse set.

Preferred shotgun libraries according to this embodiment of the invention are "representative libraries", comprising a set of tandem repeated nucleotide sequences derived from a viral pathogen of a plant or an animal.

In a particularly preferred embodiment of the invention, the shotgun library comprises cells, virus particles or bacteriophage particles comprising a diverse set of tandem-repeated nucleotide sequences which encode a diverse set of amino acid sequences, wherein the members of said diverse set of nucleotide sequences are placed operably under the control of a promoter sequence which is capable of directing the expression of said tandem-repeated nucleotide sequence in the cell.

Accordingly, the nucleotide sequence of each unit in the tandem-repeated sequence may comprise at least about 20 to 200 nucleotides in length. The use of larger fragments, particularly employing randomly sheared nucleic acid derived from viral, plant or animal genomes, is not excluded.

The introduced nucleic acid molecule is preferably in an expressible form.

By "expressible form" is meant that the subject nucleic acid molecule is presented in an arrangement such that it may be expressed in the cell, tissue, organ or whole organism, at least at the transcriptional level (i.e. it is expressed in the animal cell to yield at least an RNA product which is optionally translatable or translated to produce a recombinant peptide, oligopeptide or polypeptide molecule).

By way of exemplification, in order to obtain expression of the dispersed nucleic acid molecule or foreign nucleic acid molecule in the cell, tissue or organ of interest, a synthetic gene or a genetic construct comprising said synthetic gene is produced, wherein said synthetic gene comprises a nucleotide sequence as described *supra* in operable connection with a promoter sequence which is capable of regulating expression therein. Thus, the subject nucleic acid molecule will be operably connected to one or more regulatory elements sufficient for eukaryotic transcription to occur.

Reference herein to a "gene" or "genes" is to be taken in its broadest context and includes:

(i) a classical genomic gene consisting of transcriptional and/or translational regulatory sequences and/or a coding region and/or non-translated sequences (i.e. introns, 5'- and 3'- untranslated sequences, or DNA and RNA viruses); and/or

(ii) mRNA or cDNA corresponding to the coding regions (i.e. exons) or 5'- and 3'- untranslated sequences of the gene; and/or

(iii) a structural region corresponding to the coding regions (i.e. exons) optionally further comprising untranslated sequences and/or a heterologous promoter sequence which consists of transcriptional and/or translational regulatory regions capable of conferring expression characteristics on said structural region.

Thus, "gene" includes a nucleotide sequence coding for RNA other than mRNA.

The term "gene" is also used to describe synthetic or fusion molecules encoding all or part of a functional product, in particular a sense or antisense RNA product or a peptide, oligopeptide or polypeptide or a biologically-active protein.

The term "synthetic gene" refers to a non-naturally occurring gene as hereinbefore defined which preferably comprises at least one or more transcriptional and/or translational regulatory sequences operably linked to a structural gene sequence.

The term "structural gene" shall be taken to refer to a nucleotide sequence which is capable of being transcribed to produce RNA and optionally, encodes a peptide, oligopeptide, polypeptide or biologically active protein molecule. Those skilled in the art will be aware that not all RNA is capable of being translated into a peptide, oligopeptide, polypeptide or protein, for example if the RNA lacks a functional translation start signal or alternatively, if the RNA is antisense RNA. The present

invention clearly encompasses synthetic genes comprising nucleotide sequences which are not capable of encoding peptides, oligopeptides, polypeptides or biologically-active proteins. In particular, the present inventors have found that such synthetic genes may be advantageous in modifying target gene expression in cells, tissues or organs of a prokaryotic or eukaryotic organism.

The term "structural gene region" refers to that part of a synthetic gene which comprises a dispersed nucleic acid molecule or foreign nucleic acid molecule as described herein which is expressed in a cell, tissue or organ under the control of a promoter sequence to which it is operably connected. A structural gene region may comprise one or more dispersed nucleic acid molecules and/or foreign nucleic acid molecules operably under the control of a single promoter sequence or multiple promoter sequences. Accordingly, the structural gene region of a synthetic gene may comprise a nucleotide sequence which is capable of encoding an amino acid sequence or is complementary thereto. In this regard, a structural gene region which is used in the performance of the instant invention may also comprise a nucleotide sequence which encodes an animo acid sequence yet lacks a functional translation initiation codon and/or a functional translation stop codon and, as a consequence, does not comprise a complete open reading frame. In the present context, the term "structural gene region" also extends to a non-coding nucleotide sequences, such as 5'- upstream or 3'-downstream sequences of a gene which would not normally be translated in a eukaryotic cell which expresses said gene.

Accordingly, in the context of the present invention, a structural gene region may also comprise a fusion between two or more open reading frames of the same or different genes. In such embodiments, the invention may be used to modulate the expression of one gene, by targeting different non-contiguous regions thereof or alternatively, to simultaneously modulate the expression of several different genes, including different genes of a multigene family. In the case of a fusion nucleic acid molecule which is non-endogenous to the animal cell and in particular comprises two or more nucleotide sequences derived from a viral pathogen, the fusion may provide the added advantage of conferring simultaneous immunity or protection

against several pathogens, by targeting the expression of genes in said several pathogens. Alternatively or in addition, the fusion may provide more effective immunity against any pathogen by targeting the expression of more than one gene of that pathogen.

The optimum number of structural gene sequences which may be involved in the synthetic gene of the present invention will vary considerably, depending upon the length of each of said structural gene sequences, their orientation and degree of identity to each other. For example, those skilled in the art will be aware of the inherent instability of palindromic nucleotide sequences *in vivo* and the difficulties associated with constructing long synthetic genes comprising inverted repeated nucleotide sequences, because of the tendency for such sequences to recombine *in vivo*. Notwithstanding such difficulties, the optimum number of structural gene sequences to be included in the synthetic genes of the present invention may be determined empirically by those skilled in the art, without any undue experimentation and by following standard procedures such as the construction of the synthetic gene of the invention using recombinase-deficient cell lines, reducing the number of repeated sequences to a level which eliminates or minimises recombination events and by keeping the total length of the multiple structural gene sequence to an acceptable limit, preferably no more than 5-10kb, more preferably no more than 2-5kb and even more preferably no more than 0.5-2.0kb in length. Alternatively, as explained above, a stuffer fragment can be inserted between copies forming the palindrome.

Wherein the structural gene region comprises more than one dispersed nucleic acid molecule or foreign nucleic acid molecule it shall be referred to herein as a "multiple structural gene region" or similar term. The present invention clearly extends to the use of multiple structural gene regions which preferably comprise a direct repeat sequence, inverted repeat sequence or interrupted palindrome sequence of a particular structural gene, dispersed nucleic acid molecule or foreign nucleic acid molecule, or a fragment thereof.

Each dispersed or foreign nucleic acid molecule contained within the multiple

structural gene unit of the subject synthetic gene may comprise a nucleotide sequence which is substantially identical to a different target gene in the same organism. Such an arrangement may be of particular utility when the synthetic gene is intended to provide protection against a pathogen in a cell, tissue or organ, in particular a viral pathogen, by modifying the expression of viral target genes. For example, the multiple structural gene may comprise nucleotide sequences (i.e. two or more dispersed or foreign nucleic acid molecules) which are substantially identical to two or more target genes selected from the list comprising DNA polymerase, RNA polymerase, Nia protease, and coat protein or other target gene which is essential for viral infectivity, replication or reproduction. However, it is preferred with this arrangement that the structural gene units are selected such that the target genes to which they are substantially identical are normally expressed at approximately the same time (or later) in an infected cell, tissue or organ as (than) the multiple structural gene of the subject synthetic gene is expressed under control of the promoter sequence. This means that the promoter controlling expression of the multiple structural gene will usually be selected to confer expression in the cell, tissue or organ over the entire life cycle of the virus when the viral target genes are expressed at different stages of infection.

As with the individual sequence units of a dispersed or foreign nucleic acid molecule, the individual units of the multiple structural gene may be spatially connected in any orientation relative to each other, for example head-to-head, head-to-tail or tail-to-tail and all such configurations are within the scope of the invention.

For expression in eukaryotic cells, the synthetic gene generally comprises, in addition to the nucleic acid molecule of the invention, a promoter and optionally other regulatory sequences designed to facilitate expression of the dispersed nucleic acid molecule or foreign nucleic acid molecule.

Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences of a classical genomic gene, including the TATA box which is required for accurate transcription initiation, with

or without a CCAAT box sequence and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner. A promoter is usually, but not necessarily, positioned upstream or 5', of a structural gene region, the expression of which it regulates. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of transcription of the gene.

In the present context, the term "promoter" is also used to describe a synthetic or fusion molecule, or derivative which confers, activates or enhances expression of a nucleic acid molecule in a cell.

Preferred promoters may contain additional copies of one or more specific regulatory elements, to further enhance expression of the sense molecule and/or to alter the spatial expression and/or temporal expression of said sense molecule. For example, regulatory elements which confer copper inducibility may be placed adjacent to a heterologous promoter sequence driving expression of a sense molecule, thereby conferring copper ion inducibility on the expression of said molecule.

Placing a dispersed or foreign nucleic acid molecule under the regulatory control of a promoter sequence means positioning the said molecule such that expression is controlled by the promoter sequence. Promoters are generally positioned 5' (upstream) to the genes that they control. In the construction of heterologous promoter/structural gene combinations it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting, i.e., the gene from which the promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting, i.e., the genes from which it is derived. Again, as is known in the art, some variation in this distance

can also occur.

Examples of promoters suitable for use in the synthetic genes of the present invention include viral, fungal, bacterial, animal and plant derived promoters capable of functioning in plant, animal, insect, fungal, yeast or bacterial cells. The promoter may regulate the expression of the structural gene component constitutively, or differentially with respect to the cell, the tissue or organ in which expression occurs or, with respect to the developmental stage at which expression occurs, or in response to external stimuli such as physiological stresses, or pathogens, or metal ions, or antibiotics, amongst others.

Preferably, the promoter is capable of regulating expression of a nucleic acid molecule in a eukaryotic cell, tissue or organ, at least during the period of time over which the target gene is expressed therein and more preferably also immediately preceding the commencement of detectable expression of the target gene in said cell, tissue or organ.

Accordingly, strong constitutive promoters are particularly preferred for the purposes of the present invention or promoters which may be induced by virus infection or the commencement of target gene expression.

Plant-operable and animal-operable promoters are particularly preferred for use in the synthetic genes of the present invention. Examples of preferred promoters include the bacteriophage T7 promoter, bacteriophage T3 promoter, SP6 promoter, *lac* operator-promoter, *tac* promoter, SV40 late promoter, SV40 early promoter, RSV-LTR promoter, CMV IE promoter, CaMV 35S promoter, SCSV promoter, SCBV promoter and the like.

In consideration of the preferred requirement for high-level expression which coincides with expression of the target gene or precedes expression of the target gene, it is highly desirable that the promoter sequence is a constitutive strong promoter such as the CMV-IE promoter or the SV40 early promoter sequence, the SV40 late promoter sequence, the CaMV 35S promoter, or the SCBV promoter,

amongst others. Those skilled in the art will readily be aware of additional promoter sequences other than those specifically described.

In the present context, the terms "in operable connection with" or "operably under the control" or similar shall be taken to indicate that expression of the structural gene region or multiple structural gene region is under the control of the promoter sequence with which it is spatially connected; in a cell, tissue, organ or whole organism.

In a preferred embodiment of the invention, a structural gene region (i.e. dispersed nucleic acid molecule or foreign nucleic acid molecule) or multiple structural gene region is placed operably in connection with a promoter orientation relative to the promoter sequence, such that when it is transcribed an RNA product is synthesized which is capable of encoding a polypeptide product of the target gene or a fragment thereof.

However, the present invention is not to be limited to the use of such an arrangement and the invention clearly extends to the use of synthetic genes and genetic constructs wherein the a structural gene region or multiple structural gene region is placed in the "antisense" orientation relative to the promoter sequence, such that at least a part of the RNA transcription product thereof is complementary to the RNA encoded by the target gene or a fragment thereof.

Clearly, as the dispersed nucleic acid molecule, foreign nucleic acid molecule or multiple structural gene region comprises tandem direct and/or inverted repeat sequences of the target gene, all combinations of the above-mentioned configurations are encompassed by the invention.

In an alternative embodiment of the invention, the structural gene region or multiple structural gene region is operably connected to both a first promoter sequence and a second promoter sequence, wherein said promoters are located at the distal and proximal ends thereof such that at least one unit of said structural gene region or multiple structural gene region is placed in the "sense" orientation

relative to the first promoter sequence and in the "antisense" orientation relative to the second promoter sequence. According to this embodiment, it is also preferred that the first and second promoters be different, to prevent competition there between for cellular transcription factors which bind thereto. The advantage of this arrangement is that the effects of transcription from the first and second promoters in reducing target gene expression in the cell may be compared to determine the optimum orientation for each nucleotide sequence tested.

The synthetic gene preferably contains additional regulatory elements for efficient transcription, for example a transcription termination sequence.

The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which signals termination of transcription. Terminators are 3'-non-translated DNA sequences which may contain a polyadenylation signal, which facilitates the addition of polyadenylate sequences to the 3'-end of a primary transcript. Terminators active in plant cells are known and described in the literature. They may be isolated from bacteria, fungi, viruses, animals and/or plants or synthesized *de novo*.

As with promoter sequences, the terminator may be any terminator sequence which is operable in the cells, tissues or organs in which it is intended to be used.

Examples of terminators particularly suitable for use in the synthetic genes of the present invention include the SV40 polyadenylation signal, the HSV TK polyadenylation signal, the CYC1 terminator, ADH terminator, SPA terminator, nopaline synthase (NOS) gene terminator of *Agrobacterium tumefaciens*, the terminator of the Cauliflower mosaic virus (CaMV) 35S gene, the *zein* gene terminator from *Zea mays*, the Rubisco small subunit gene (SSU) gene terminator sequences, subclover stunt virus (SCSV) gene sequence terminators, any *rho*-independent *E.coli* terminator, or the *lacZ* alpha terminator, amongst others.

In a particularly preferred embodiment, the terminator is the SV40 polyadenylation signal or the HSV TK polyadenylation signal which are operable in animal cells,

tissues and organs, octopine synthase (OCS) or nopaline synthase (NOS) terminator active in plant cells, tissues or organs, or the *lacZ* alpha terminator which is active in prokaryotic cells.

Those skilled in the art will be aware of additional terminator sequences which may be suitable for use in performing the invention. Such sequences may readily be used without any undue experimentation.

Means for introducing (i.e. transfecting or transforming) cells with the synthetic genes described herein or a genetic construct comprising same are well-known to those skilled in the art.

In a further alternative embodiment, a genetic construct is used which comprises two or more structural gene regions or multiple structural gene regions wherein each of said structural gene regions is placed operably under the control of its own promoter sequence. As with other embodiments described herein, the orientation of each structural gene region may be varied to maximise its modulatory effect on target gene expression.

According to this embodiment, the promoters controlling expression of the structural gene unit are preferably different promoter sequences, to reduce competition there between for cellular transcription factors and RNA polymerases. Preferred promoters are selected from those referred to *supra*.

Those skilled in the art will know how to modify the arrangement or configuration of the individual structural genes as described *supra* to regulate their expression from separate promoter sequences.

The synthetic genes described *supra* are capable of being modified further, for example by the inclusion of marker nucleotide sequences encoding a detectable marker enzyme or a functional analogue or derivative thereof, to facilitate detection of the synthetic gene in a cell, tissue or organ in which it is expressed. According to this embodiment, the marker nucleotide sequences will be present in

a translatable format and expressed, for example as a fusion polypeptide with the translation product(s) of any one or more of the structural genes or alternatively as a non-fusion polypeptide.

Those skilled in the art will be aware of how to produce the synthetic genes described herein and of the requirements for obtaining the expression thereof, when so desired, in a specific cell or cell-type under the conditions desired. In particular, it will be known to those skilled in the art that the genetic manipulations required to perform the present invention may require the propagation of a genetic construct described herein or a derivative thereof in a prokaryotic cell such as an *E. coli* cell or a plant cell or an animal cell.

The synthetic genes of the present invention may be introduced to a suitable cell, tissue or organ without modification as linear DNA in the form of a genetic construct, optionally contained within a suitable carrier, such as a cell, virus particle or liposome, amongst others. To produce a genetic construct, the synthetic gene of the invention is inserted into a suitable vector or episome molecule, such as a bacteriophage vector, viral vector or a plasmid, cosmid or artificial chromosome vector which is capable of being maintained and/or replicated and/or expressed in the host cell, tissue or organ into which it is subsequently introduced.

Accordingly a further aspect of the invention provides a genetic construct which at least comprises the synthetic gene according to any one or more of the embodiments described herein and one or more origins of replication and/or selectable marker gene sequences.

Genetic constructs are particularly suitable for the transformation of a eukaryotic cell to introduce novel genetic traits thereto, in addition to the provision of resistance characteristics to viral pathogens. Such additional novel traits may be introduced in a separate genetic construct or, alternatively on the same genetic construct which comprises the synthetic genes described herein. Those skilled in the art will recognise the significant advantages, in particular in terms of reduced

genetic manipulations and tissue culture requirements and increased cost-effectiveness, of including genetic sequences which encode such additional traits and the synthetic genes described herein in a single genetic construct.

Usually, an origin of replication or a selectable marker gene suitable for use in bacteria is physically-separated from those genetic sequences contained in the genetic construct which are intended to be expressed or transferred to a eukaryotic cell, or integrated into the genome of a eukaryotic cell.

In a particularly preferred embodiment, the origin of replication is functional in a bacterial cell and comprises the pUC or the ColE1 origin or alternatively the origin of replication is operable in a eukaryotic cell, tissue and more preferably comprises the 2 micron (2• m) origin of replication or the SV40 origin of replication.

As used herein, the term "selectable marker gene" includes any gene which confers a phenotype on a cell in which it is expressed to facilitate the identification and/or selection of cells which are transfected or transformed with a genetic construct of the invention or a derivative thereof.

Suitable selectable marker genes contemplated herein include the ampicillin-resistance gene (Amp^r), tetracycline-resistance gene (Tc^r), bacterial kanamycin-resistance gene (Kan^r), the zeocin resistance gene (Zeocin is a drug of bleomycin family which is trademark of InVitrogen Corporation), the *AURI-C* gene which confers resistance to the antibiotic aureobasidin A, phosphinothricin-resistance gene, neomycin phosphotransferase gene (*npt*II), hygromycin-resistance gene, • -glucuronidase (GUS) gene, chloramphenicol acetyltransferase (CAT) gene, green fluorescent protein- encoding gene or the luciferase gene, amongst others.

Preferably, the selectable marker gene is the *npt*II gene or Kan^r gene or green fluorescent protein (GFP)-encoding gene.

Those skilled in the art will be aware of other selectable marker genes useful in the performance of the present invention and the subject invention is not limited by the

nature of the selectable marker gene.

The present invention extends to all genetic constructs essentially as described herein, which include further genetic sequences intended for the maintenance and/or replication of said genetic construct in prokaryotes or eukaryotes and/or the integration of said genetic construct or a part thereof into the genome of a eukaryotic cell or organism.

As with dispersed or foreign nucleic acid molecules, standard methods described *supra* may be used to introduce synthetic genes and genetic constructs into the cell, tissue or organ for the purposes of modulating the expression of the target gene, for example liposome-mediated transfection or transformation, transformation of cells with attenuated virus particles or bacterial cells, cell mating, transformation or transfection procedures known to those skilled in the art or described by Ausubel *et al.* (1992).

Additional means for introducing recombinant DNA into plant tissue or cells include, but are not limited to, transformation using $CaCl_2$ and variations thereof, in particular the method described by Hanahan (1983), direct DNA uptake into protoplasts (Krens *et al*, 1982; Paszkowski *et al*, 1984), PEG-mediated uptake to protoplasts (Armstrong *et al*, 1990) microparticle bombardment, electroporation (Fromm *et al.*, 1985), microinjection of DNA (Crossway *et al.*, 1986), microparticle bombardment of tissue explants or cells (Christou *et al*, 1988; Sanford, 1988), vacuum-infiltration of tissue with nucleic acid, or in the case of plants, T-DNA-mediated transfer from *Agrobacterium* to the plant tissue as described essentially by An *et al.*(1985), Herrera-Estrella *et al.* (1983a, 1983b, 1985).

For microparticle bombardment of cells, a microparticle is propelled into a cell to produce a transformed cell. Any suitable ballistic cell transformation methodology and apparatus can be used in performing the present invention. Exemplary apparatus and procedures are disclosed by Stomp *et al.* (U.S. Patent No. 5,122,466) and Sanford and Wolf (U.S. Patent No. 4,945,050). When using ballistic transformation procedures, the genetic construct may incorporate a

plasmid capable of replicating in the cell to be transformed.

Examples of microparticles suitable for use in such systems include 1 to 5 μm gold spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

In a further embodiment of the present invention, the synthetic genes and genetic constructs described herein are adapted for integration into the genome of a cell in which it is expressed. Those skilled in the art will be aware that, in order to achieve integration of a genetic sequence or genetic construct into the genome of a host cell, certain additional genetic sequences may be required. In the case of plants, left and right border sequences from the T-DNA of the *Agrobacterium tumefaciens* Ti plasmid will generally be required.

The present invention further extends to an isolated cell, tissue or organ comprising the synthetic gene described herein or a genetic construct comprising same. The present invention extends further to regenerated tissues, organs and whole organisms derived from said cells, tissues and organs and to propagules and progeny thereof.

For example, plants may be regenerated from transformed plant cells or tissues or organs on hormone-containing media and the regenerated plants may take a variety of forms, such as chimeras of transformed cells and non-transformed cells; clonal transformants (e.g., all cells transformed to contain the expression cassette); grafts of transformed and untransformed tissues (e.g., a transformed root stock grafted to an untransformed scion in citrus species). Transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, a first generation (or T1) transformed plants may be selfed to give homozygous second generation (or T2) transformed plants, and the T2 plants further propagated through classical breeding techniques.

The present invention is further described with reference to the following non-

limiting Examples.

EXAMPLE 1

Generic techniques

1. Tissue culture manipulations

(a) Adherent cell lines

Adherent cell monolayers were grown in medium consisting of either DMEM (Life Technologies) supplemented with 10% v/v FBS (TRACE Biosciences or Life Technologies) or RPMI 1640 Medium (Life Technologies) supplemented with 10% v/v FBS. Cells were grown in incubators at 37°C in an atmosphere containing 5% v/v CO_2.

During the course of these experiments it was frequently necessary to passage the cell monolayer. To achieve this, the monolayers were washed twice with 1 x PBS (Sigma) and then treated with trypsin-EDTA (Life Technologies) for 5 min at 37°C. The volumes of trypsin-EDTA used for such manipulations were typically 20 µl, 100 µl, 500 µl, 1 ml and 2 ml for 96-well, 48-well, 6-well, T25 and T75 vessels, respectively. The action of the trypsin-EDTA was stopped with an equal volume of growth medium. The cells were suspended by trituration. A 1/5 volume of the cell suspension was then transferred to a new vessel containing growth medium. Tissue culture medium volumes were typically 192 µl/well for 96-well tissue culture plates, 360 µl/well for 48-well tissue culture plates, 3.8 ml/well for 6-well tissue culture plates, 9.6 ml for T25 and 39.2 ml for T75 tissue culture vessels.

Cell suspensions were counted after resuspending in an appropriate volume of DMEM, 10% v/v FBS. An aliquot, typically 100 µl, was transferred to a haemocytometer (Hawksley) and cell numbers counted microscopically.

(b) Non-adherent cells

Non-adherent cells were grown in growth medium similarly to adherent cell lines.

As in the case of adherent monolayers, frequent changes of tissue culture vessels were necessary. For T25 and T75 vessels, the cell suspension was removed to 50 ml sterile plastic tubes (Falcon) and centrifuged for 5 min at 500 x g and 4°C. The supernatant was then discarded and the cell pellet suspended in growth medium. The cell suspension was then placed into a new tissue culture vessel. For 96-well, 48-well, and 6-well vessels, the vessels were centrifuged for 5 min at 500 x g and 4°C. The supernatant was then aspirated away from the cell pellet and the cells suspended in growth medium. The cells were then transferred to a new tissue culture vessel. Tissue culture media volumes were typically 200 µl/well for 96-well tissue culture plates, 400 µl/well for 48-well tissue culture plates, 4 ml/well for 6-well tissue culture plates, 11 ml for T25 and 40 ml for T75 tissue culture vessels.

Passaging the cell suspensions was achieved in the following manner: Cells were centrifuged for 5 min at 500 x g and 4°C and suspended in 5 ml growth medium. Then 0.5 ml (T25) or 1.0 ml (T75) of the cell suspension was transferred to a new vessel containing growth medium. For cells in 96-well, 48-well, and 6-well plates, a 1/5 volume of cells was transferred to the corresponding wells of a new vessel containing 4/5 volume of growth medium.

Cells were counted as described for adherent cells.

2. Protocol for freezing and thawing cells

Adherent monolayers were washed twice with 1 x PBS and then treated with trypsin-EDTA for 5 min at 37°C. Non-adherent cells were centrifuged for 5 min at 500 x g and 4°C. The cells were suspended by trituration and transferred to storage medium consisting of DMEM or RPMI 1640 supplemented with 20% v/v FBS and 10% v/v dimethylsulfoxide (Sigma). The concentration of cells was determined by haemocytometer counting and diluted to 10^5 cells per ml. Aliquots

were transferred to 1.5 ml cryotubes (Nunc) and the tubes were placed in a Cryo 1°C Freezing Container (Nalgene) containing propan-2-ol (BDH) and cooled slowly to -70°C. The tubes of cells were then stored at -70°C. Reanimation of frozen cells was achieved by warming the tubes to 0°C on ice then transferring the cells to a T25 flask containing DMEM and 20% v/v FBS and incubating at 37°C in an atmosphere of 5% v/v CO_2.

3. Cloning of cell lines

Adherent and non-adherent mammalian cell lines were transfected with plasmid vectors containing expression constructs to target specific genes of interest. Stable, transformed colonies were selected over a period of 2–3 weeks using cell growth medium (either DMEM, 10% v/v FBS or RPMI 1640, 10% v/v FBS) supplemented with geneticin. Individual colonies were cloned to establish clonal lines of transfected cells.

(a) Conditioned medium

Conditioned media were prepared by overlaying 20–30%-confluent monolayers of cells grown in a T75 vessel with 40 ml of DMEM, 10% v/v FBS. Vessels were incubated at 37°C in 5% v/v CO_2 for 24 hr, after which the growth medium was transferred to a sterile 50 ml tube (Falcon) and centrifuged at 500 x g. The medium was passed through a 0.45 μm filter and decanted to a fresh sterile tube for use as conditioned medium.

(b) Adherent cells

Individual lines were cloned from discrete colonies as follows: First, the medium was removed from an individual well of a 6-well tissue culture vessel and the cell colonies washed twice with 2 ml of 1 x PBS. Individual colonies were then detached from the plastic culture vessel with a sterile plastic pipette tip and moved to a 96-well plate containing 200 μl of conditioned medium supplemented with geneticin. The vessel was incubated at 37°C in 5% v/v CO_2 for approximately 72

hr. Individual wells were examined microscopically for growing colonies and the medium replaced with fresh growth medium. When the monolayer of each stable line had reached about 90% confluency it was transferred in successive steps until the stable, transformed line was housed in a T25 tissue culture vessel. At this point, aliquots of each stable cell line were frozen for long term maintenance.

(c) Non-adherent cells

Non-adherent cells were cloned by dilution cloning. Cell concentration was determined microscopically using a haemocytometer slide and cells were diluted to 10 cells per ml in conditioned medium. Single wells of 96-well tissue culture vessels were seeded with 200 μl of the diluted cells and the plates were incubated at 37°C in 5% v/v CO_2 for 48 hr. Wells were inspected microscopically and those containing a single colony, arising from a single cell, were defined as clonal cell lines. The medium was removed and replaced with 200 μl of fresh conditioned medium and cells incubated at 37°C in 5% v/v CO_2 for 48 hr. After this time, conditioned medium was replaced with 200 μl of DMEM, 10% v/v FBS and 1.5 mg/l geneticin and cells again incubated at 37°C in 5% v/v CO_2. Colonies were allowed to expand in successive steps, with medium changes every 48 hr, until the stable, transformed lines were housed in T25 tissue culture vessels. At this point, aliquots of each stable cell line were frozen for long term maintenance

4. Southern blot analysis of mammalian genomic DNA

For all subsequent examples, Southern blot analyses of genomic DNA were carried out according to the following protocol.

A T75 tissue culture vessel containing 40 ml of DMEM or RPMI 1640, 10% v/v FBS was seeded with 4 x 10^6 cells and incubated at 37°C in 5% v/v CO_2 for 24 hr.

(a) Adherent cells

Medium was decanted and 5 ml of 1 x PBS was added to the T75 flask to wash

the monolayer by gentle rocking then the PBS was decanted. The wash was repeated and the monolayer overlaid with 2 ml of 1 x PBS/1 x Trypsin-EDTA, ensuring even coverage of the monolayer by gentle rocking. The flask was incubated at 37°C in 5% v/v CO_2 until the monolayer separated from the flask, then 2 ml of medium with 10% v/v FBS was added. The suspended cells were transferred into a 10 ml capped tube to which was added 3 ml of ice-cold 1 x PBS. The tubewas inverted several times to mix and the cells were collected by centrifugation at 500 x g for 10 min at 4°C. The supernatant was decanted and the pellet suspended in 5 ml of ice-cold 1 x PBS by gentle vortexing and a sample was counted (≈2 x 10^8). The cells were collected by centrifugation at 500 x g for 10 min at 4°C and the supernatant decanted.

(b) Non-adherent cells

The cell suspension is transferred into a 50 ml Falcon tube, centrifuged at 500 x g for 10 min at 4°C and the supernatant decanted. The pellet was suspended in 5 ml ice-cold 1 x PBS by gentle vortexing and the cells collected by centrifugation at 500 x g for 10 min at 4°C. The supernatant was decanted and the pellet was resuspended in 5 ml of ice-cold 1 x PBS by gentle vortexing and a sample counted (≈2 x 10^8). The cells were collected by centrifugation at 500 x g for 10 min at 4°C and the supernatant decanted.

(c) DNA extraction and analysis

Genomic DNA, for both adherent and non-adherent cell lines, was extracted using the Qiagen Genomic DNA extraction kit (Cat No. 10243) according to the supplier's instructions. The concentration of genomic DNA was determined from absorbance at 260 nm using a Beckman model DU64 photospectrometer.

Genomic DNA (10 μg) was digested with appropriate restriction endonucleases and buffer in a volume of 200 μl at 37°C for approximately 16 hr. Following digestion, 20 μl of 3M sodium acetate, pH 5.2, and 500 μl of absolute ethanol were

added to the digest and the solution mixed by vortexing and chilled at -20°C for 2 hr. DNA was recovered by centrifugation at 10,000 x g for 30 min at 4°C. The supernatant was removed and the DNA pellet rinsed with 500 µl of 70% v/v ethanol, the pellet air-dried and the DNA dissolved in 20 µl of water.

Gel loading buffer (0.25% w/v bromophenol blue (Sigma), 0.25% w/v xylene cyanol FF (Sigma), 15% w/v Ficoll Type 400 (Pharmacia)) (5 µl) was added to the digested DNA and the mixture transferred to a well of a 0.7% w/v agarose/TAE gel containing 0.5 µg/ml of ethidium bromide. The DNA fragments were electrophoresed through the gel at 14 volts for approximately 16 hr. An appropriate DNA size marker was included in a parallel lane.

The gel was soaked in 1.5 M NaCl, 0.5 M NaOH then in 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.0. The DNA fragments were then capillary blotted to Hybond NX (Amersham) membrane and fixed by UV cross-linking (Bio Rad GS Gene Linker).

The Hybond membrane was rinsed in sterile water and stained in 0.4% v/v methylene blue in 300mM sodium acetate, pH 5.2, for 5 min to visualize the transferred genomic DNA. The membrane was then rinsed twice in sterile water, destained in 40% v/v ethanol then rinsed in sterile water.

The membrane was placed in a Hybaid bottle with 5 ml of pre-hybridization solution (6 x SSPE, 5 x Denhardt's reagent, 0.5% w/v SDS, 100 µg/ml denatured, fragmented herring sperm DNA) and pre-hybridized at 60°C for approximately 14 hr in a hybridization oven with constant rotation (6 rpm).

Probe (25 ng) was labelled with [α^{32}P]-dCTP (specific activity 3000 Ci/mmol) using the Megaprime DNA labelling system as per the supplier's instructions (Amersham Cat. No. RPN1606). Labelled probe was passed through a G50 Sephadex Quick Spin (trademark) column (Roche, Cat. No. 1273973) to remove unincorporated nucleotides as per the supplier's instructions.

The heat-denatured labelled probe was added to 2 ml of hybridization buffer (6 x

SSPE, 0.5% w/v SDS, 100 μg/ml denatured, fragmented herring sperm DNA) pre-warmed to 60°C. The pre-hybridization buffer was decanted and replaced with 2 ml of pre-warmed hybridization buffer containing the labelled probe. The membrane was hybridized at 60°C for approximately 16 hr in a hybridization oven with constant rotation (6 rpm).

The hybridization buffer containing the probe was decanted and the membrane subjected to sequential washes as follows:

2 x SSC, 0.5% w/v SDS for 5 min at room temperature;

2 x SSC, 0.1% w/v SDS for 15 min at room temperature;

0.1 x SSC, 0.5% w/v SDS for 30 min at 37°C with gentle agitation;

0.1 x SSC, 0.5% w/v SDS for 1 hour at 68°C with gentle agitation; and

0.1 x SSC for 5 min at room temperature with gentle agitation.

Washing duration at 68°C varied based on the amount of radioactivity detected with a hand-held Geiger counter.

The damp membrane was wrapped in plastic wrap and exposed to X-ray film (Curix Blue HC-S Plus, AGFA) for 24–48 hr and the film developed to visualize bands of probe hybridized to genomic DNA.

5. Immunofluorescent labelling of cultured cells

Glass microscope cover slips (12 mm x 12 mm) were flamed with ethanol then submerged in 2 ml growth medium, two per well, in six-well plates. Cells were added to wells in 1–2 ml medium to give a density after 16 hr growth such that cells remain isolated (200,000 to 500,000 per well depending on size and growth rate). Without removing the cover slips from the wells, the medium was aspirated

and cells were washed with PBS. For fixation, cells were treated for 1 hr with 4% w/v paraformaldehyde (Sigma) in PBS then washed three times with PBS. Fixed cells were permeabilized with 0.1% v/v Triton X-100 (Sigma) in PBS for 5 min then washed three times with PBS. Cells on cover slips were blocked on one drop (about 100 μl) of 0.5% w/v bovine serum albumin Fraction V (BSA, Sigma) for 10 min. Cover slips were then placed for at least 1 h on 25 μl drops of primary mouse monoclonal antibody which had been diluted 1/100 in 0.5% v/v BSA in PBS. Cells on cover slips were then washed three times with 100 μl of 0.5% v/v BSA in PBS for about 3 min each before being placed for 30 min to 1 hr on 25 μl drops of Alexa Fluor (registered trademark) 488 goat anti-mouse IgG conjugate (Molecular Probes) secondary antibody diluted 1/100 in 0.5% v/v BSA in PBS. Cells on cover slips were then washed three times with PBS. Cover slips were mounted on glass microscope slides, three to a slide, in glycerol/DABCO (25 mg/ml DABCO (1,4-diazabicyclo(2.2.2)octane (Sigma D 2522)) in 80% v/v glycerol in PBS) and examined with a 100x oil immersion objective under UV illumination at 500–550 nm.

EXAMPLE 2

Genetic constructs comprising BEV polymerase gene sequences linked to the CMV promoter sequence and/or the SV40L promoter sequence

1. Commercial Plasmids

Plasmid pBluescript II (SK+)

Plasmid pBluescript II (SK+) is commercially available from Stratagene and comprises the LacZ promoter sequence and *lac*Z-alpha transcription terminator, with a multiple cloning site for the insertion of structural gene sequences therein. The plasmid further comprises the ColE1 and f1 origins of replication and ampicillin-resistance gene.

Plasmid pSVL

Plasmid pSVL is commercially-obtainable from Pharmacia and serves as a source of the SV40 late promoter sequence. The nucleotide sequence of pSVL is also publicly available as GenBank Accession Number U13868.

Plasmid pCR2.1

Plasmid pCR2.1 is commercially available from Invitrogen and comprises the LacZ promoter sequence and *lac*Z-α transcription terminator, with a cloning site for the insertion of structural gene sequences there between. Plasmid pCR2.1 is designed to clone nucleic acid fragments by virtue of the A-overhang frequently synthesized by *Taq* polymerase during the polymerase chain reaction. PCR fragments cloned in this fashion are flanked by two EcoRI sites. The plasmid further comprises the ColE1 and f1 origins of replication and kanamycin-resistance and ampicillin-resistance genes.

Plasmid pEGFP-N1 MCS

Plasmid pEGFP-N1 MCS (Figure 1; Clontech) contains the CMV IE promoter operably connected to an open reading frame encoding a red-shifted variant of wild-type green fluorescent protein (GFP; Prasher *et al.*, 1992; Chalfie *et al.*, 1994; Inouye and Tsuji, 1994), which has been optimised for brighter fluorescence. The specific GFP variant encoded by pEGFP-N1 MCS has been disclosed by Cormack *et al.* (1996). Plasmid pEGFP-N1 MCS contains a multiple cloning site comprising *Bgl*I and *Bam*HI sites and many other restriction endonuclease cleavage sites, located between the CMV IE promoter and the GFP open reading frame. Structural genes cloned into the multiple cloning site will be expressed at the transcriptional level if they lack a functional translation start site, however such structural gene sequences will not be expressed at the protein level (i.e. translated). Structural gene sequences inserted into the multiple cloning site which comprise a functional translation start site will be expressed as GFP fusion polypeptides if they are cloned in-frame with the GFP-encoding sequence. The

plasmid further comprises an SV40 polyadenylation signal downstream of the GFP open reading frame to direct proper processing of the 3'-end of mRNA transcribed from the CMV-IE promoter sequence. The plasmid further comprises the SV40 origin of replication functional in animal cells; the neomycin-resistance gene comprising SV40 early promoter (SV40 EP in Figure 1) operably connected to the neomycin/kanamycin-resistance gene derived from Tn5 (Kan/neo in Figure 1) and the HSV thymidine kinase polyadenylation signal (HSV TK poly (A) in Figure 1), for selection of transformed cells on kamanycin, neomycin or G418; the pUC19 origin of replication which is functional in bacterial cells (pUC Ori in Figure 1); and the f1 origin of replication for single-stranded DNA production (f1 Ori in Figure 1).

2. Expression cassettes

Plasmid pCMV.cass

Plasmid pCMV.cass (Figure 2) is an expression cassette for driving expression of a structural gene sequence under control of the CMV-IE promoter sequence. Plasmid pCMV.cass was derived from pEGFP-N1 MCS by deletion of the GFP open reading frame as follows: Plasmid pEGFP-N1 MCS was digested with *Pin*AI and *Not* I, blunt-ended using *Pfu*I polymerase and then re-ligated. Structural gene sequences are cloned into pCMV.cass using the multiple cloning site, which is identical to the multiple cloning site of pEGFP-N1 MCS, except it lacks the *Pin*AI site.

Plasmid pCMV.SV40L.cass

Plasmid pCMV.SV40L.cass (Figure 3) comprises the synthetic poly A site and the SV40 late promoter sequence from plasmid pCR.SV40L (Figure 4), sub-cloned as a *Sal* I fragment, into the *Sal* I site of plasmid pCMV.cass (Figure 2), such that the CMV-IE promoter and SV40 late promoter sequences are capable of directing transcription in the same direction. Accordingly, the synthetic poly(A) site at the 5' end of the SV40 promoter sequence is used as a transcription terminator for structural genes expressed from the CMV IE promoter in this plasmid, which also

provides for the insertion of said structural gene via the multiple cloning site present between the SV40 late promoter and the synthetic poly(A) site (Figure 5). The multiple cloning sites are located behind the CMV-IE and SV40 late promoters, including *Bam*HI and *Bgl*I sites.

Plasmid pCMV.SV40LR.cass

Plasmid pCMV.SV40LR.cass (Figure 4) comprises the SV40 late promoter sequence derived from plasmid pCR.SV40L, sub-cloned as a *Sal*I fragment into the *Sal*I site of the plasmid pCMV.cass (Figure 2), such that the CMV-IE or the SV40 late promoter may drive transcription of a structural gene or a multiple structural gene unit, in the sense or antisense orientation, as desired. A multiple cloning site is positioned between the opposing CMV- IE and SV40 late promoter sequences in this plasmid to facilitate the introduction of a structural gene sequence. In order for expression of a structural gene sequence to occur from this plasmid, it must be introduced with its own transcription termination sequence located at the 3' end, because there are no transcription termination sequences located between the opposing CMV- IE and SV40 late promoter sequences in this plasmid. Preferably, the structural gene sequence or multiple structural gene unit which is to be introduced into pCMV.SV40LR.cass will comprise both a 5' and a 3' polyadenylation signal as follows:

(i) where the structural gene sequence or multiple structural gene unit is to be expressed in the sense orientation from the CMV IE promoter sequence and/or in the antisense orientation from the SV40 late promoter, the 5' polyadenylation signal will be in the antisense orientation and the 3' polyadenylation signal will be in the sense orientation; and

(ii) where the structural gene sequence or multiple structural gene unit is to be expressed in the antisense orientation from the CMV IE promoter sequence and/or in the sense orientation from the SV40 late promoter, the 5' polyadenylation signal will be in the sense orientation and the 3' polyadenylation signal will be in the antisense orientation.

Alternatively or in addition, suitably-oriented terminator sequences may be placed at the 5'-end of the CMV and SV40L promoters, as shown in Figure 4.

Alternatively, plasmid pCMV.SV40LR.cass is further modified to produce a derivative plasmid which comprises two polyadenylation signals located between the CMV IE and SV40 late promoter sequences, in approriate orientations to facilitate expression of any structural gene located therebetween in the sense or antisense orientation from either the CMV IE promoter or the SV40 promoter sequence. The present invention clearly encompasses such derivatives.

Alternatively appropriately oriented terminators could be placed upstream of the CMV and SV40L promoters such that transcriptional termination could occur after readthrough of each of the two promoters in the antisense orientation.

3. Intermediate Constructs

Plasmid pCR.Bgl-GFP-Bam

Plasmid pCR.Bgl-GFP-Bam (Figure 5) comprises an internal region of the GFP open reading frame derived from plasmid pEGFP-N1 MCS (Figure 1) placed operably under the control of the lacZ promoter. To produce this plasmid, a region of the GFP open reading frame was amplified from pEGFP-N1 MCS using the amplification primers Bgl-GFP (SEQ ID NO:1) and GFP-Bam (SEQ ID NO:2) and cloned into plasmid pCR2.1. The internal GFP-encoding region in plasmid pCR.Bgl-GFP-Bam lacks functional translational start and stop codons.

Plasmid pBSII(SK+).EGFP

Plasmid pBSII(SK+).EGFP (Figure 6) comprises the EGFP open reading frame derived from plasmid pEGFP-N1 MCS (Figure 1) placed operably under the control of the *lac*Z promoter. To produce this plasmid, the EGFP encoding region of pEGFP-N1 MCS was excised as a *Not*1/*Xho*1 fragment and cloned into the *Not*1/*Xho*1 cloning sites of plasmid pBluescript II (SK+).

Plasmid pCMV.EGFP

Plasmid pCMV.EGFP (Figure 7) is capable of expressing the EGFP structural gene under the control of the CMV-IE promoter sequence. To produce this plasmid the EGFP sequence from plasmid pBSII(SK+).EGFP was excised as *Bam*HI/*Sac*I fragment and cloned into the *Bgl*II/*Sac*I sites of plasmid pCMV.cass (Figure 2).

Plasmid pCR.SV40L

Plasmid pCR.SV40L (Figure 8) comprises the SV40 late promoter derived from plasmid pSVL (GenBank Accession No. U13868; Pharmacia), cloned into pCR2.1 (Stratagene). To produce this plasmid, the SV40 late promoter was amplified using the primers SV40-1 (SEQ ID NO:3) and SV40-2 (SEQ ID NO:4) which comprise *Sal* I cloning sites to facilitate sub-cloning of the amplified DNA fragment into pCMV.cass. The primer also contains a synthetic poly (A) site at the 5' end, such that the amplicification product comprises the synthetic poly(A) site at the 5' end of the SV40 promoter sequence.

Plasmid pCR.BEV.1

The BEV RNA-dependent RNA polymerase coding region was amplified as a 1,385 bp DNA fragment from a full-length cDNA clone encoding same, using primers designated BEV-1 (SEQ ID NO:5) and BEV-2 (SEQ ID NO:6), under standard amplification conditions. The amplified DNA contained a 5'-*Bgl* II restriction enzyme site, derived from the BEV-1 primer sequence and a 3'*Bam*HI restriction enzyme site, derived from the BEV-2 primer sequence. Additionally, as the BEV-1 primer sequence contains a translation start signal 5'-ATG-3' engineered at positions 15-17, the amplified BEV polymerase structural gene comprises the start site in-frame with BEV polymerase-encoding nucleotide sequences, Thus, the amplified BEV polymerase structural gene comprises the ATG start codon immediately upstream (ie. juxtaposed) to the BEV polymerase-encoding sequence. There is no translation stop codon in the amplified DNA. This

plasmid is present as Figure 9.

Plasmid pCR.BEV.2

The complete BEV polymerase coding region was amplified from a full-length cDNA clone encoding same, using primers BEV-1 and BEV-3. Primer BEV-3 comprises a *Bam*HI restriction enzyme site at positions 5 to 10 inclusive and the complement of a translation stop signal at positions 11 to 13. As a consequence, an open reading frame comprising a translation start signal and translation stop signal, contained between the *Bgl* II and *Bam*HI restriction sites. The amplified fragment was cloned into pCR2.1 (Stratagene) to produce plasmid pCR2.BEV.2 (Figure 10).

Plasmid pCR.BEV.3

A non-translatable BEV polymerase structural gene was amplified from a full-length BEV polymerase cDNA clone using the amplification primers BEV-3 (SEQ ID NO:7) and BEV-4 (SEQ ID NO:8). Primer BEV-4 comprises a *Bgl*I cloning site at positions 5-10 and sequences downstream of this *Bgl*I site are homologous to nucleotide sequences of the BEV polymerase gene. There is no functional ATG start codon in the amplified DNA product of primers BEV-3 and BEV-4. The BEV polymerase is expressed as part of a polyprotein and, as a consequence, there is no ATG translation start site in this gene. The amplified DNA was cloned into plasmid pCR2.1 (Stratagene) to yield plasmid pCR.BEV.3 (Figure 11).

Plasmid pCMV.EGFP.BEV2

Plasmid pCMV.EGFP.BEV2 (Figure 12) was produced by cloning the BEV polymerase sequence from pCR.BEV.2 as a BglII/BamHI fragment into the BamHI site of pCMV.EGFP.

4. Control Plasmids

Plasmid pCMV.BEV.2

Plasmid pCMV.BEV.2 (Figure 13) is capable of expressing the entire BEV polymerase open reading frame under the control of CMV-IE promoter sequence. To produce pCMV.BEV.2, the BEV polymerase sequence from pCR.BEV.2 was sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bgl*II/*Bam*HI-digested pCMV.cass (Figure 2).

Plasmid pCMV.BEV.3

Plasmid pCMV.BEV.3 (Figure 14) expresses a non-translatable BEV polymerase structural gene in the sense orientation under the control of the CMV-IE promoter sequence. To produce pCMV.BEVnt, the BEV polymerase sequence from pCR.BEV.3 was sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bgl*II/*Bam*HI-digested pCMV.cass (Figure 2).

Plasmid pCMV.VEB

Plasmid pCMV.VEB (Figure 15) expresses an antisense BEV polymerase mRNA under the control of the CMV-IE promoter sequence. To produce plasmid pCMV.VEB, the BEV polymerase sequence from pCR.BEV.2 was sub-cloned in the antisense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bgl*II/*Bam*HI-digested pCMV.cass (Figure 2).

Plasmid pCMV.BEV.GFP

Plasmid pCMV.BEV.GFP (Figure 16) was constructed by cloning the GFP fragment from pCR.Bgl-GFP-Bam as a *Bgl*II/*Bam*HI fragment into *Bam*HI-digested pCMV.BEV.2. This plasmid serves as a control in some experiments and also as an intermediate construct.

Plasmid pCMV.BEV.SV40L.0

Plasmid pCMV.BEV.SV40L.0 (Figure 17) comprises a translatable BEV polymerase structural gene derived from plasmid pCR.BEV.2 inserted in the sense orientation between the CMV-IE promoter and the SV40 late promoter sequences of plasmid pCMV.SV40L.cass. To produce plasmid pCMV.BEV.SV40L-O, the BEV polymerase structural gene was sub-cloned as a *Bgl*II-to-*Bam*HI fragment into *Bgl*II-digested pCMV.SV40L.cass DNA.

Plasmid pCMV.O.SV40L.BEV

Plasmid pCMV.O.SV40L.BEV (Figure 18) comprises a translatable BEV polymerase structural gene derived from plasmid pCR.BEV.2 cloned downstream of tandem CMV-IE promoter and SV40 late promoter sequences present in plasmid pCMV.SV40L.cass. To produce plasmid pCMV.O.SV40L.BEV, the BEV polymerase structural gene was sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.SV40L.cass DNA.

Plasmid pCMV.O.SV40L.VEB

Plasmid pCMV.O.SV40L.VEB (Figure 19) comprises an antisense BEV polymerase structural gene derived from plasmid pCR.BEV.2 cloned downstream of tandem CMV-IE promoter and SV40 late promoter sequences present in plasmid pCMV.SV40L.cass. To produce plasmid pCMV.O.SV40L.VEB, the BEV polymerase structural gene was sub-cloned in the antisense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.SV40L.cass DNA.

5. Test Plasmids

Plasmid pCMV.BEVx2

Plasmid pCMV.BEVx2 (Figure 20) comprises a direct repeat of a complete BEV polymerase open reading frame under the control of the CMV-IE promoter

sequence. In eukaryotic cells at least, the open reading frame located nearer the CMV-IE promoter is translatable. To produce pCMV.BEVx2, the BEV polymerase structural gene from plasmid pCR.BEV.2 was sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.BEV.2, immediately downstream of the BEV polymerase structural gene already present therein.

Plasmid pCMV.BEVx3

Plasmid pCMV.BEVx3 (Figure 21) comprises a direct repeat of three complete BEV polymerase open reading frames under the control of the CMV-1E promoter. To produce pCMV.BEVx3, the BEV polymerase fragment from pCR.BEV.2 was cloned in the sense orientation as a *Bgl*II/*Bam*HI fragment into the *Bam*HI site of pCMV.BEVx2, immediately downstream of the BEV polymerase sequences already present therein.

Plasmid pCMV.BEVx4

Plasmid pCMV.BEVx4 (Figure 22) comprises a direct repeat of four complete BEV polymerase open reading frames under the control of the CMV-1E promoter. To produce pCMV.BEVx4, the BEV polymerase fragment from pCR.BEV.2 was cloned in the sense orientation as a *Bgl*II/*Bam*HI fragment into the *Bam*HI site of pCMV.BEVx3, immediately downstream of the BEV polymerase sequences already present therein.

Plasmid pCMV.BEV.SV40L.BEV

Plasmid pCMV.BEV.SV40L.BEV(Figure 23) comprises a multiple structural gene unit comprising two BEV polymerase structural genes placed operably and separately under control of the CMV-IE promoter and SV40 late promoter sequences. To produce plasmid pCMV.BEV.SV40L.BEV, the translatable BEV polymerase structural gene present in pCR.BEV.2 was sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment behind the SV40 late promoter sequence present in *Bam*HI-digested pCMV.BEV.SV40L-O.

Plasmid pCMV.BEV.SV40L.VEB

Plasmid pCMV.BEV.SV40L.VEB (Figure 24) comprises a multiple structural gene unit comprising two BEV polymerase structural genes placed operably and separately under control of the CMV-IE promoter and SV40 late promoter sequences. To produce plasmid pCMV.BEV.SV40L.VEB, the translatable BEV polymerase structural gene present in pCR.BEV.2 was sub-cloned in the antisense orientation as a *Bgl*II-to-*Bam*HI fragment behind the SV40 late promoter sequence present in *Bam*HI-digested pCMV.BEV.SV40L-O. In this plasmid, the BEV polymerase structural gene is expressed in the sense orientation under control of the CMV-IE promoter to produce a translatable mRNA, whilst the BEV polymerase structural gene is also expressed under control of the SV40 promoter to produce an antisense mRNA species.

Plasmid pCMV.BEV.GFP.VEB

Plasmid pCMV.BEV.GFP.VEB (Figure 25) comprises a BEV structural gene inverted repeat or palindrome, interrupted by the insertion of a GFP open reading frame (stuffer fragment) between each BEV structural gene sequence in the inverted repeat. To produce plasmid pCMV.BEV.GFP.VEB, the GFP stuffer fragment from pCR.Bgl-GFP-Bam was first sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.BEV.2 to produce an intermediate plasmid pCMV.BEV.GFP wherein the BEV polymerase-encoding and GFP-encoding sequences are contained within the same 5'-*Bgl*II-to-*Bam*HI-3' fragment. The BEV polymerase structural gene from pCMV.BEV.2 was then cloned in the antisense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.BEV.GFP. The BEV polymerase structural gene nearer the CMV-IE promoter sequence in plasmid pCMV.BEV.GFP.VEB is capable of being translated, at least in eukaryotic cells.

Plasmid pCMV.EGFP.BEV2.PFG

Plasmid pCMV.EGFP.BEV2.PFG (Figure 26) comprise a GFP palindrome,

interrupted by the insertion of a BEV polymerase sequence between each GFP structural gene in the inverted repeat. To produce this plasmid the GFP fragment from pCR.Bgl-GFP-Bam was cloned as a *Bgl*II/*Bam*HI fragment into the *Bam*HI site of pCMV.EGFP.BEV2 in the antisense orientation relative to the CMV promoter.

Plasmid pCMV.BEV.SV40LR

Plasmid pCMV.BEV.SV40LR (Figure 27) comprises a structural gene comprising the entire BEV polymerase open reading frame placed operably and separately under control of opposing CMV-IE promoter and SV40 late promoter sequences, thereby potentially producing BEV polymerase transcripts at least from both strands of the full-length BEV polymerase structural gene. To produce plasmid pCMV.BEV.SV40LR, the translatable BEV polymerase structural gene present in pCR.BEV.2 was sub-cloned, as a *Bgl*II-to-*Bam*HI fragment, into the unique *Bgl*II site of plasmid pCMV.SV40LR.cass, such that the BEV open reading frame is present in the sense orientation relative to the CMV-IE promoter sequence.

Those skilled in the art will recognise that it is possible to generate a plasmid wherein the BEV polymerase fragment from pCR.BEV.2 is inserted in the antisense orientation, relative to the CMV IE promoter sequence, using this cloning strategy. The present invention further encompasses such a genetic construct.

EXAMPLE 3

Genetic constructs comprising the porcine α-1,3-galactosyltransferase (Galt) structural gene sequence or sequences operably connected to the CMV promoter sequence and/or the SV40L promoter sequence

1. Commercial Plasmids

Plasmid pcDNA3

Plasmid pcDNA3 is commercially available from Invitrogen and comprises the CMV-IE promoter and BGHpA transcription terminator, with multiple cloning sites for the insertion of structural gene sequences there between. The plasmid further comprises the ColE1 and fl origins of replication and neomycin-resistance and ampicillin-resistance genes.

2. Intermediate plasmids

Plasmid pcDNA3.Galt

Plasmid pcDNA3.Galt (BresaGen Limited, South Australia, Australia; Figure 28) is plasmid pcDNA3 (Invitrogen) and comprises the cDNA sequence encoding porcine gene alpha-1,3-galactosyltransferase (Galt) operably under the control of the CMV-IE promoter sequence such that it is capable of being expressed therefrom. To produce plasmid pcDNA3.Galt, the porcine gene alpha-1,3-galactosyltransferase cDNA was cloned as an *Eco*RI fragment into the *Eco*RI cloning site of pcDNA3. The plasmid further comprises the ColE1 and fl origins of replication and the neomycin and ampicillin-resistance genes.

3. Control Plasmids

Plasmid pCMV.Galt

Plasmid pCMV.Galt (Figure 29) is capable of expressing the Galt structural gene

under the control of the CMV-IE promoter sequence. To produce plasmid pCMV.Galt, the Galt sequence from plasmid pcDNA3.Galt was excised as an *Eco*RI fragment and cloned in the sense orientation into the *Eco*RI site of plasmid pCMV.cass (Figure 2).

Plasmid pCMV.EGFP.Galt

Plasmid pCMV.EGFP.Galt (Figure 30) is capable of expressing the Galt structural gene as a Galt fusion polypeptide under the control of the CMV-IE promoter sequence. To produce plasmid pCMV.EGFP.Galt, the Galt sequence from pCMV.Galt (Figure 29) was excised as a *Bgl*II/*Bam*HI fragment and cloned into the *Bam*HI site of pCMV.EGFP.

Plasmid pCMV.Galt.GFP

Plasmid pCMV.Galt.GFP (Figure 31) was produced by cloning the Galt cDNA as an EcORI fragment from pCDNA3 into EcoRI-digested pCMV.EGFP in the sense orientation. This plasmid serves as both a control and construct intermediate.

Plasmid pCMV.Galt.SV40L.0

The plasmid pCMV.Galt.SV40L.0 (Figure 32) comprises a Galt structural gene cloned downstream of the CMV promoter present in pCMV.SV40L.cass. To produce the plasmid the Galt cDNA fragment from pCMV.Galt was cloned as a *Bgl*II/*Bam*HI into *Bgl*II-digested pCMV.SV40L.cass in the sense orientation.

Plasmid pCMV.O.SV40L.tlaG

The plasmid pCMV.O.SV40L.tlaG (Figure 33) comprises a Galt structural gene clones in an antisense orientation downstream of the SV40L promoter present in pCMV.SV40L.cass. To produce this plasmid the Galt cDNA fragment from pCMV.Galt was cloned as a *Bgl*II/*Bam*HI into *Bam*HI-digested pCMV.SV40L.cass in the antisense orientation.

Plasmid pCMV.O.SV40L.Galt

The plasmid pCMV.O.SV40L.Galt (Figure 34) comprises a Galt structural gene cloned downstream of the SV40L promoter present in pCMV.SV40L.cass. To produce the plasmid the Galt cDNA fragment from pCMV.Galt was cloned as a *Bgl*II/*Bam*HI into *Bam*HI-digested pCMV.SV40L.cass in the sense orientation.

4. Test Plasmids

Plasmid pCMV.Galtx2

Plasmid pCMV.Galtx2 (Figure 35) comprises a direct repeat of a Galt open reading frame under the control of the CMV-IE promoter sequence. In eukaryotes cells at least, the open reading frame located nearer the CMV-IE promoter is translatable. To produce pCMV.Galtx2, the Galt structural gene from pCMV.Galt was excised as a *Bgl*II/*Bam*HI fragment and cloned in the sense orientation into the *Bam*HI cloning site of pCMV.Galt.

Plasmid pCMV.Galtx4

Plasmid pCMV.Galtx4 (Figure 36) comprises a quadruple direct repeat of a Galt open reading frame under the control of the CMV-IE promoter sequence. In eukaryotes cells at least, the open reading frame located nearer the CMV-IE promoter is translatable. To produce pCMV.Galtx4, the Galtx2 sequence from pCMV.Galtx2 was excised as a *Bgl*II/*Bam*HI fragment and cloned in the sense orientation into the *Bam*HI cloning site of pCMV.Galtx2.

Plasmid pCMV.Galt.SV40L.Galt

The plasmid pCMV.Galt.SV40L.Galt (Figure 37) is designed to express two sense transcripts of Galt, one driven by the CMV promoter, the other by the SV40L promoter. To produce the plasmid a Galt cDNA fragment from pCMV.Galt was cloned as a *Bgl*II/*Bam*HI fragment into *Bgl*II-digested pCMV.O.SV40.Galt in the

sense orientation.

Plasmid pCMV.Galt.SV40L.tlaG

The plasmid pCMV.Galt.SV40.tlaG (Figure 38) is designed to express a sense transcript of Galt driven by the CMV promoter and an antisense transcript driven by the SV40L promoter. To produce the plasmid a Galt cDNA fragment from pCMV.Galt was cloned as a BglII/BamHI fragment into BglII-digested pCMV.O.SV40.talG in the sense orientation.

Plasmid pCMV.Galt.GFP.tlaG

Plasmid pCMV.Galt.GFP.tlaG (Figure 39) comprise a Galt palindrome, interrupted by the insertion of a GFP sequence between each Galt structural gene in the inverted repeat. To produce this plasmid the *Bgl*II/*Bam*HI Galt cDNA fragment from pCMV.Galt was cloned into the *Bam*HI site of pCMV.Galt.GFP in the antisense relative to the CMV promoter.

Plasmid pCMV.EGFP.Galt.PFG

The plasmid pCMV.EGFP.Galt.PFG (Figure 40) comprises a GFP palindrome, interrupted by the insertion of a Galt sequence between each GFP structural gene of the inverted repeat, expression of which is driven by the CMV promoter. To produce this plasmid the Galt sequences from pCMV.Galt were cloned as a BglII/BamHI fragment into BamHI-digested pCMV.EGFP in the sense orientation to produce the intermediate pCMV.EGFP.Galt (not shown); following this further GFP sequences from pCR.Bgl-pCMV.EGFP.Galt in the antisense orientation.

Plasmid pCMV.Galt.SV40LR

The plasmid pCMV.Galt.SV40LR (Figure 41) is designed to express Galt cDNA sequences cloned between the opposing CMV and SV40L promoters in the expression cassette pCMV.SV40LR.cass. To produce this plasmid Galt

sequences from pCMV.Galt were cloned as a BglII/BamHI fragment in BglII-digested pCMV.SV40LR.cass in the sense orientation relative to the 35S promoter.

EXAMPLE 4

Genetic constructs comprising PVY Nia sequences operably linked to the35S promoter sequence and/or the SCBV promoter sequence

1. Binary vector

Plasmid pART27

Plasmid pART27 is a binary vector, specifically designed to be compatible with the pART7 expression cassette. It contains bacterial origins of replication for both *E. coli* and *Agrobacterium tumefaciens*, a spectinomycin resistance gene for bacterial selection, left and right T-DNA borders for transfer of DNA from Agrobacterium to plant cells and a kanamycin resistance cassette to permit selection of transformed plant cells. The kanamycin resistance cassette is located between the T-DNA borders, pART27 also contains a unique NotI restriction site which permits cloning of constructs prepared in vectors such as pART7 to be cloned between the T-DNA borders. Construction of pART27 is described in Gleave, AP (1992).

When cloning NotI inserts into this vector, two insert orientations can be obtained. In all the following examples the same insert orientation, relative to the direction of the 35S promoter in the described pART7 constructs was chosen; this was done to minimise any experimental artefacts that may arise from comparing different constructs with different insert orientations.

2. Commercial plasmids

Plasmid pBC (KS-)

Plasmid pBC (KS-) is commercially available from Stratagene and comprises the

LacZ promoter sequence and lacZ-alpha transcription terminator, with a multiple cloning site for the insertion of structural gene sequences therein. The plasmid further comprises the ColE1 and fl origins of replication and a chloroamphenicol-resistance gene.

Plasmid pSP72

Plasmid pSP72 is commercially available from Promega and contains a multiple cloning site for the insertion of structural gene sequences therein. The plasmid further comprises the ColE1 origin of replication and an ampicillin-resistance gene.

3. Expression cassettes

Plasmid pART7

Plasmid pART7 is an expression cassette designed to drive expression of sequences cloned behind the 35S promoter. It contains a polylinker to assist cloning and a region of the octipine synthase terminator. The 35S expression cassette is flanked by two Not I restriction sites which permits cloning into binary expression vectors, such as pART27 which contains a unique NotI site. Its construction as described in Gleave, AP (1992), a map is shown in Figure 42.

Plasmid pART7.35S.SCBV.cass

Plasmid p35S.CMV.cass was designed to express two separate gene sequences cloned into a single plasmid. To create this plasmid, sequences corresponding to the nos terminator and the SCBV promoter were amplified by PCR then cloned in the polylinker of pART7 between the 35S promoter and OCS.

The resulting plasmid has the following arrangement of elements:

35S promoter - polylinker 1 - NOS terminator - SCBV promoter - polylinker 2 - OCS terminator.

Expression of sequences cloned into polylinker 1 is controlled by the 35S promoter, expression of sequences cloned into polylinker 2 is controlled by the SCBV promoter.

The NOS terminator sequences were amplified from the plasmid pAHC27 (Christensen and Quail, 1996) using the two oligonucleotides;

NOS 5' (forward primer; SEQ ID 9)

5'-GGATTCCCGGGACGTCGCGAATTTCCCCCGATCGTTC-3'; and

NOS 3' (reverse primer; SEQ ID 10)

5'-CCATGGCCATATAGGCCCGATCTAGTAACATAG-3'

Nucleotide residues 1 to 17 for NOS 5' and 1 to 15 for NOS 3' represent additional nucleotides designed to assist in construct preparation by adding additional restriction sites. For NOS 5' these are BamHI, SmaI, AatII and the first 4 bases of an NruI site, for NOS 3' these are NcoI and SfiI sites. The remaining sequences for each oligonucleotide are homologous to the 5' and 3' ends respectively of NOS sequences in pAHC 27.

The SCBV promoter sequences were amplified from the plasmid pScBV-20 (Tzafir *et al*, 1998) using the two oligonucleotides:

SCBV 5': 5'-CCATGGCCTATATGGCCATTCCCCACATTCAAG-3' (SEQ ID NO:11); and

SCBV 3': 5'-AACGTTAACTTCTACCCAGTTCCAGAG-3' (SEQ ID NO:12)

Nucleotide residues 1 to 17 of SCBV 5' encode NcoI and SfiI restriction sites designed to assist in construct preparation, the remaining sequences are homologous to upstream sequences of the SCMV promoter region. Nucleotide residues 1 to 9 of SCBV 3' encode Psp10461 and HpaI restriction sites designed

to assist in construct preparation, the remaining sequences are homologous to the reverse and complement of sequences near the transcription initiation site of SCBV.

Sequences amplified from pScBV-20 using PCR and cloned into pCR2.1 (Invitrogen) to produce pCR.NOS and pCR.SCBV respectively. Sma I/Sfil cut pCR.NOS and Sfil/Hpal cut pCR.SCBV were ligated into Sma I cut pART7 and a plasmid with a suitable orientation was chosen and designated pART7.35S.SCBV.cass, a map of this construct is shown in Figure 70.

4. Intermediate constructs

Plasmid pBC.PVY

A region of the PVY genome was amplified by PCR using reverse-transcribed RNA isolated from PVY-infected tobacco as a template using standard protocols and cloned into a plasmid pGEM 3 (Stratagene), to create pGEM.PVY. A SalI/HindIII fragment from pGEM.PVY, corresponding to a SalI/HindIII fragment positions 1536-2270 of the PVY strain O sequence (Acc. No D12539, Genbank), was then subcloned into the plasmid pBC (Stratagene Inc.) to create pBC.PVY (Figure 44).

Plasmid pSP72.PVY

Plasmid pSP72.PVY was prepared by inserting an EcoRI/SalI fragment from pBC.PVY into EcoRI/SalI cut pSP72 (Promega). This construct contains additional restriction sites flanking the PVY insert which were used to assist subsequent manipulations. A map of this construct is shown in Figure 45.

Plasmid ClapBC.PVY

Plasmid ClapBC.PVY was prepared by inserting a ClaI/SalI fragment from pSP72.PVY into ClaI/Sal I cutpBC (Stratagene). This construct contains additional

restriction sites flanking the PVY insert which were used to assist subsequent manipulations. A map of this construct is shown in Figure 46.

Plasmid pBC.PVYx2

Plasmid pBC.PVYx2 contains two direct head-to-tail repeats of the PVY sequences derived from pBC.PVY. The plasmid was generated by cloning an AccI/ClaI PVY fragment from pSP72.PVY into AccI cut pBC.PVY and is shown in Figure 47.

Plasmid pSP72.PVYx2

Plasmid pSP72.PVYx2 contains two direct head-to-tail repeats of the PVY sequences derived from pBC.PVY. The plasmid was generated by cloning an AccI/ClaI PVY fragment from pBc.PVY into AccI cut pSP72.PVY and is shown in Figure 48.

Plasmid pBC.PVYx3

Plasmid pBC.PVYx3 contains three direct head-to-tail repeats of the PVY sequences derived from pBC.PVY. The plasmid was prepared by cloning an AccI/ClaI PVY fragment from pSP72.PVY into AccI cut pBC.PVYx2 and is shown in Figure 49.

Plasmid pBC.PVYx4

Plasmid pBC.PVYx4 contains four direct head-to-tail repeats of the PVY sequences derived from pBC.PVY. The plasmid was prepared by cloning the direct repeat of PVY sequences from pSP72.PVYx2 as an AccI/ClaI fragment into AccI cut pBC.PVYx2 and is shown in Figure 50.

Plasmid pBC.PVY.LNYV

All attempts to create direct palindromes of PVY sequences failed, *presumably*

such sequence arrangements are unstable in commonly used E. coli cloning hosts. Interrupted palindromes however proved stable.

To create interrupted palindromes of PVY sequences a "stuffer" fragment of approximately 360 bp was inserted into Cla pBV.PVY downstream of the PVY sequences. The stuffer fragment was made as follows:

A clone obtained initially from a cDNA library prepared from lettuce necrotic yellows virus (LNYV) genomic RNA (Deitzgen *et al*, 1989), known to contain the 4b gene of the virus, was amplified by PCR using the primers:

LNYV 1:5'-ATGGGATCCGTTATGCCAAGAAGAAGGA-3' (SEQ ID NO:13); and

LNYV 2:5'-TGTGGATCCCTAACGGACCCGATG-3' (SEQ ID NO:14)

The first 9 nucleotide of these primers encode a *Bam*HI site, the remaining nucleotides are homologous to sequences of the LNYV 4b gene.

Following amplification, the fragment was cloned into the EcoRI site of pCR2.1 (Stratagene). This EcoRI fragment was cloned into the EcoRI site of Cla pBC.PVY to create the intermediate plasmid pBC.PVY.LNYV which is shown in Figure 51.

Plasmid pBC.PVY.LNYV.PVY

The plasmid pBC.PVY.LNYV.YVP contains an interrupted direct repeat of PVY sequences. to create this plasmid a HpaI/HincII fragment from pSP72 was cloned into SmaI-digested pBC.PVY.LNYV and a plasmid containing the sense orientation isolated, a map of this construct is shown in Figure 52.

Plasmid pBC.PVY.LNYV.YVPΔ

The plasmid pBV.PVY.LNYV.YVPΔ contains a partial interrupted palindrome of PVY sequences. One arm of the palindrome contains all the PVY sequences from pBC.PVY, the other arm contains part of the sequences from PVY, corresponding

to sequences between the EcoRV and HincII sites of pSP72.PVY. To create this plasmid an EcoRV/HincII fragment from pSP72.PVY was cloned into SmaI-digested pBC.PVY.LNYV and a plasmid containing the desired orientation isolated, a map of this construct is shown in Figure 53.

Plasmid pBC.PVY.LNYV.YVP

The plasmid pBC.PVY.LNYV.YVP contains an interrupted palindrome of PVY sequences. To create this plasmid a HpaI/HincII fragment from pSP72. was cloned into Sma-digested pBC.PVY.LNYV and a plasmid containing the antisense orientation isolated, a map of this construct is shown in Figure 54.

5. Control plasmids

Plasmids pART7.PVY & pART7.PVY

Plasmid pART7.PVY (Figure 55) was designed to express PVY sequences driven by the 35S promoter. This plasmid serves as a control construct in these experiments, against which all other constructs was compared. To generate this plasmid a ClaI/AccI fragment from ClapBC.PVY was cloned into ClaI-digested pART7 and a plasmid with expected to express a sense PVY sequence with respect to the PVY genome, was selected. Sequences consisting of the 35S promoter, PVY sequences and the OCS terminator were excised as a NotI fragment and cloned into NotI-digested pART27, a plasmid with the desired insert orientation was selected and designated pART27.

Plasmids pART7.35S.PVY.SCBV.O & pART27.35S.PVY.SCBV.O

Plasmid pART7.35S.PVY.SCBV.0 (Figure 56) was designed to act as a control for co-expression of multiple constructs from a single plasmid in transgenic plants. The 35S promoter was designed to express PVY sense sequences, whilst the SCBV promoter was empty. To generate this plasmid, the PVY fragment from Cla pBC.PVY was cloned as a XhoI/EcoRI fragment into XhoI/EcoRI-digested

pART7.35S.SCBV.cass to create p35S.PVY.SCBV.O. Sequences consisting of the 35S promoter driving sense PVY sequences and the NOS terminator and the SCBV promoter and OCS terminator were excised as a NotI fragment and cloned into pART27, a plasmid with the desired insert orientation was isolated and designated pART27.35S.PVY.SCBV.O.

Plasmids pART7.35S.O.SCBV.PVY & pART27.35S.O.SCBV.PVY

Plasmid pART27.35S.O.SCBV.PVY (Figure 57) was designed to act as an additional control for co-expression of multiple constructs from a single plasmid in transgenic plants. No expressible sequences were cloned behind the 35S promoter, whilst the SCBV promoter drove expression of a PVY sense fragment. To generate this plasmid, the PVY fragment from Cla pBC.PVY was cloned as a ClaI fragment into ClaI-digested pART7.35S.SCBV.cass, a plasmid containing PVY sequences in a sense orientation was isolated and designated p35S.O.SCBV.PVY. Sequences, consisting of the 35S promoter and NOS terminator, the SCBV promoter driving sense PVY sequences and the OCS terminator were excised as a NotI fragment and cloned into pART27, a plasmid with the desired insert orientation was isolated and designated pART27.35S.O.SCBV.PVY.

Plasmids pART7.35S.O.SCBV.YVP & pART7.35S.O.SCBV.YVP

Plasmid pART7.35S.O.SCBV.YVP (Figure 58) was designed to act as an additional control for co-expression of multiple constructs from a single plasmid in transgenic plants. No expressible sequences were cloned behind the 35S promoter, whilst the SCBV promoter drove expression of a PVY antisense fragment. To generate this plasmid, the PVY fragment from Cla pBC.PVY was cloned as a ClaI fragment into ClaI-digested p35S.SCBV.cass, a plasmid containing PCY sequences in an antisense orientation was isolated and designated p35S.O.SCBV.YVP. Sequences, consisting of the 35S promoter and NOS terminator, the SCBV promoter driving sense PVY sequences and the OCS terminator were excised as a NotI fragment and cloned into pART27, a plasmid

with the desired insert orientation was isolated and designated pART27.35S.O.SCBV.YVP.

6. Test plasmids

Plasmids pART7.PVYx2 & pART27.PVYx2

Plasmid pART7.PVYx2 (Figure 59) was designed to express a direct repeat of PVY sequences driven by the 35S promoter in transgenic plants. To generate this plasmid, direct repeats from pBC.PVYx2 were cloned as a XhoI/BamHI fragment into XhoI/BamHI cut pART7. Sequences consisting of the 35 S promoter, direct repeats of PVY and the OCS terminator were excised as a NotI fragment from pART7.PVYx2 and cloned into NotI-digested pART27, a plasmid with the desired insert orientation was selected and designated pART27.PVYx2.

Plasmids pART7.PVYx3 & pART27.PVYx3

Plasmid pART7.PVYx3 (Figure 60) was designed to express a direct repeat of three PVY sequences driven by the 35S promoter in transgenic plants. To generate this plasmid, direct repeats from pBC.PVYx3 were cloned as a XhoI/BamHI fragment into XhoI/BamHI cut pART7. Sequences consisting of the 35S promoter, direct repeats of PVY and OCS terminator were excised as a NotI fragment from pART.PVYx3 and cloned into NotI-digested pART27, a plasmid with the desired insert orientation was selected and designated pART27.PVYx3.

Plasmids pART7.PVYx4 & pART27.PVYx4

Plasmid pART7.PVYx4 (Figure 61) was designed to express a direct repeat of four PVY sequences driven by the 35S promoter in transgenic plants. To generate this plasmid, direct repeats from pBC.PVYx4 were cloned as a XhoI/BamHI fragment into xhoI/BamHI cut pART7. Sequences consisting of the 35S promoter, direct repeats of PVY and the OCS terminator were excised as a NotI fragment from pART7.PVYx3 and cloned into NotI-digested pART27, a plasmid with the desired

insert orientation was selected and designated pART27.PVYx3.

Plasmids pART7.PVY.LNYV.PVY & pART27.PVY.LNYV.PVY

Plasmid pART7.PVY.LNYV.PVY (Figure 62) was designed to express the interrupted direct repeat of PVY sequences driven by the 35S promoter in transgenic plants. This construct was prepared by cloning the interrupted direct repeat of PVY from pBC.PVY.LNYV.PVY as a XhoI/XbaI fragment into pART7 digested with XhoI/XbaI. Sequences consisting of the 35S promoter, the interrupted direct repeat of PVY sequences and the OCS terminator were excised from pART7.PVY.LNYV.PVY as a NotI fragment and cloned into NotI-digested pART27, a plasmid with the desired insert orientation was selected and designated pART27.PVY.LNYV.PVY.

Plasmids pART7.PVY.LNYV.YVP$_\Delta$ & pART27.PVY.LNYV.YVP$_\Delta$

Plasmid pART7.PVY.LNYV.YVP$_\Delta$ (Figure 63) was designed to express the partial interrupted palindrome of PVY sequences driven by the 35S promoter in transgenic plants. This construct was prepared by cloning the partial interrupted palindrome of PVY sequences from pBC.PVY.LNYV.YVP$_\Delta$ as a XhoI/XbaI fragment into pART7 digested with XhoI/XbaI. Sequences consisting of the 35S promoter, the partial interrupted palindrome of PVY sequences and the OCS terminator were excised from pART7.PVY.LNYV.YVP$_\Delta$ as a NotI fragment and cloned into NotI-digested pART27, a plasmid with the desired insert orientation was selected and designated pART27.PVY.LNYV.YVP.

Plasmids pART7.PVY.LNYV.YVP & pART27.PVY.LNYV.YVP

Plasmid pART7.PVY.LNYV.YVP (Figure 64) was designed to express the interrupted palindrome of PVY sequences driven by the 35S promoter in transgenic plants. This construct was prepared by cloning the interrupted palindrome of PVY sequences from pBC.PVY.LNYV.YVP$_\Delta$ as a XhoI/XbaI fragment into pART7 digested with XhoI/XbaI. Sequences consisting of the 35S

promoter, the interrupted palindrome of PVY sequences and the OCS terminator were excised from pART7.PVY.LNYV.YVP as a NotI fragment and cloned into pART27, a plasmid with the desired insert orientation was selected and designated pART27.PVY.LNYV.YVP.

Plasmids pART7.35S.PVY.SCBV.YVP & pART27.35S.PVY.SCBV.YVP

Plasmid pART7.35S.PVY.SCBV.YVP (Figure 65) was designed to co-express sense and antisense constructs in transgenic plants. To generate this plasmid the PVY fragment from Cla pBC.PVY was cloned as a XhoI/EcoRI fragment into xhoI/EcoRI-digested p35S.SCBV.O.SCBV.YVP. Sequences, consisting of the 35S promoter driving sense PVY sequences and the NOS terminator and the SCBV promoter driving antisense PVY and the OCS terminator were excised as a NotI fragment and cloned into pART27, a plasmid with the desired insert orientation was isolated and designated pART27.35S.PVY.SCBV.YVP.

Plasmids pART7.35S.PVYx3.SCBV.YVPx3 & pART27.35S.PVYx3.SCBV.YVPx3

Plasmid pART7.35S.PVYx3.SCBV.YVPx3 (Figure 66) was designed to co-express sense and antisense repeats of PVY in transgenic plants. to generate this plasmid, the intermediate pART7.35S.O.SCBV.YVPx3 was constructed by cloning the triple direct PVY repeat from ClapBC.PVYx3 as a ClaI/AccI fragment into Cla-digested p35S.SCBV.cass and isolating a plasmid with an antisense orientation. for p35S.PVYx3.SCBV.YVPx3 the triple direct PVY repeat from Cla pBC.PVYx3 was cloned as a KpnI/SmaI fragment into KpnI/SmaI-digested p35S.O.SCBV.YVPx3 to create p35S.PVYx3.SCBV.YVPx3. Sequences including both promoters, terminators and direct PVY repeats were isolated as a NotI fragment and cloned into pART27. A plasmid with an appropriate orientation was chosen and designated pART27.35S.PVYx3.SCBV.

Plasmids pART7.PVYx3.LNYV.YVPx3 & pART27.PVYx3.LNYV.YVPx3

Plasmid pART7.PVYx3.LNYV.YVPx3 (Figure 67) was designed to express triple repeats of PVY sequences as an interrupted palindrome. To generate this plasmid an intermediate, pART7x3.PVY.LNYV.YV was constructed by cloning a PVY.LNYV.YVP fragment from pBC.PVY.LNYV.YVP as an AccI/ClaI fragment into the plasmid pART7.PVYx2. pART7.35S.PVYx3.LNYV.YVPx3, was made by cloning an additional PVY direct repeat from pBC.PVYx2 as an AccI/ClaI fragment into ClaI digested pART7x3.PVY.LNYV.YVP. Sequences from pART7.35S.PVYx3.LNYV.YVPx3, including the 35S promoter, all PVY sequences and the OCS terminator were excised as a NotI fragment and cloned into NotI-digested pART27, a plasmid with an appropriate orientation was chosen and designated pART27.35S.PVYx3.LNYV.

Plasmids pART7.PVY multi & pART27.PVY multi

Plasmid pART7.35S.PVY multi (Figure 68) was designed to express higher order direct repeats of regions of PVY sequences in transgenic plants. Higher order direct repeats of a 72 bp of the PVY Nia region from PVY were prepared by annealing two partially complementary oligonucleotides as follows:

PVY1:

5'-TAATGAGGATGATGTCCCTACCTTTAATTGGCAGAAATTTCTGTGGAAAGACAG

GGAAATCTTTCGGCATTT-3' (SEQ ID NO:15); and

PVY2:

5'-TTCTGCCAATTAAAGGTAGGGACATCATCCTCATTAAAATGCCGAAAGATT

TCCCTGTCTTTCCACAGAAAT-3' (SEQ ID NO:16)

The oligonucleotides were phosphorylated with T4 polynucleotide kinase, heated

and cooled slowly to permit self-annealing, ligated with T4 DNA ligase, end-filled with Klenow polymerase and cloned into pCR2.1 (Invitrogen). Plasmids containing multiple repeats were isolated and sequences were cloned as EcoRI fragments in a sense orientation into EcoRI-digested pART7, to create the intermediate pART7.PVY multi. to create pART27.PVY multi, the 35S promoter, PVY sequences and the OCS terminator were excised as a NotI fragment and cloned into NotI-digested pART27. A plasmid with an appropriate insert orientation was isolated and designated pART27.PVY multi.

EXAMPLE 5

Inactivation of virus gene expression in mammals

Viral immune lines are created by expressing viral sequences in stably transformed cell lines.

In particular, lytic viruses are used for this approach since cell lysis provides very simple screens and also offer the ability to directly select for potentially rare transformation events which might create viral immunity. Sub-genomic fragments derived from a simple single stranded RNA virus (Bovine enterovirus - BEV) or a complex double stranded DNA virus, Herpes Simplex Virus I (HSV I) are cloned into a suitable vector and expressed in transformed cells. Mammalian cell lines are transformed with genetic constructs designed to express viral sequences driven by the strong cytomegalovirus (CMV-IE) promoter. Sequences utilised include specific viral replicase genes. Random "shotgun" libraries comprising representative viral gene sequences, may also be used and the introduced dispersed nucleic acid molecule, to target the expression of virus sequences.

Exemplary genetic constructs for use in this procedure, comprising nucleotide sequences derived from the BEV RNA-dependent RNA polymerase gene, are presented herein.

For viral polymerase constructs, large numbers (approximately 100) of

transformed cell lines are generated and infected with the respective virus. For cells transformed with shotgun libraries very large numbers (hundreds) of transformed lines are generated and screened in bulk for viral immunity. Following virus challenge, resistant cell lines are selected and analysed further to determine the sequences conferring immunity thereon.

Resistant cell lines are supportive of the ability of the introduced nucleotide sequences to inactivate viral gene expression in a mammalian system.

Additionally, resistant lines obtained from such experiments are used to more precisely define molecular and biochemical characteristics of the modulation which is observed.

EXAMPLE 6

Induction of virus resistance in transgenic plants

Agrobacterium tumefaciens, strain LBA4404, was transformed independently with the constructs

pART27.PVY

pART27.PVYx2

pART27.PVYx3

pART27.PVYx4

pART27.PVY.LNYV.PVY

pART27.PVY.LNYV.YVPΔ

pART27.PVY.LNYV.YVP

pART27.35S.PVY.SCBV.O

pART27.35S.O.SCBV.PVY

pART27.35S.O.SCBV.YVP

pART27.35S.PVY.SCBV.YVP

pART27.35S.PVYx3.SCBV.YPVx3

pART27.PVYx3.LNYV.YVPx3

pART27.PVYx10

using tri-parental matings. DNA mini-preps from these strains were prepared and examined by restriction with NotI to ensure they contained the appropriate binary vectors.

Nicotiana tabaccum (cultivar W38) were transformed with these *Agrobacterium* strains using standard procedures. Putative transformed shoots were excised and rooted on media containing kanamycin. Under these conditions we have consistently observed that only transgenic shoots will root on kanamycin plates. Rooted shoots were transferred to soil and allowed to establish. After two to three weeks, vigorous plants with at least three sets of leaves were chosen and infected with PVY.

Viral inoculum was prepared from W38 tobacco previously infected with the virus, approximately 2 g of leaf material, showing obvious viral symptoms were ground with carbarundum in 10 ml of 100mM Na phosphate buffer (pH 7.5). the inoculum was diluted to 200 ml with additional Na phosphate buffer. Two leaves from each transgenic plant were sprinkled with carbarundum, then 0.4 ml of inoculum was applied to each leaf and leaves rubbed fairly vigorously with fingers. Using this procedure 100% of non-transgenic control plants were infected with PVY.

To assay for viral resistance and immunity transgenic plants are monitored for symptom development. The PVY strain (PVY-D, an Australian PVY isolate) gives obvious symptoms on W38 tobacco, a vein clearing symptom is readily observed on the two leaves above the inoculated leaves, subsequent leaves show uniform chlorotic lesions. Symptom development was monitored over a six week period.

Transgenic lines were described as resistant if they showed reduced viral symptoms, which manifests as a reduction in the leaf are showing chlorotic lesions. Resistance ranges from very strong resistance where only a few viral lesions are observed on a plant to weak resistance which manifects as reduced symptoms on leaves that develop late in plant growth.

Transgenic plants which showed absolutely no evidence of viral symptoms were classified as immune. To ensure these plants were immune they were re-inoculated with virus, most plants remained immune, the few that showed symptoms were re-classified as resistant.

For plant lines generated Southern blots are performed, resistance in subsequent generations is monitored to determine that resistance/immunity is transmissable. Additionally, the breadth of viral resistance is monitored by challenging lines with other PVY strains, to determine whether host range susceptibility is modified.

Results from these experiments are described in Table 2. These data indicate that constructs comprising tandem repeats of target gene sequence, either in the configuration of palindromes, interrupted palindromes as direct repeat sequences, are capable of conferring viral resistance and/or immunity in transgenic plants.

Accordingly, such inverted and/or direct repeat sequences modulate expression of the virus target gene in the transgenic plant.

Constructs combining the use of direct and inverted repeat sequences, namely pART27.35S.PVYx3.SCBV.YVPx3 and pART27.PVYx3.LNYV.YVPx3, are also useful in modulating gene expression.

EXAMPLE 7

Inactivation of Galt in animal cells

To assay for Galt inactivation, porcine PK2 cells were transformed with the relevant constructs. PK2 cells constitutively express Galt enzyme, the activity of which results in the addition of a variety of α-1,3-galactosyl groups to a range of proteins expressed on the cell surface of these cells. Cells were transformed using lipofectin and stably transformed lines were selected using genetecin.

As an initial assay cell lines were probed for the presence of the Galt-encoded epitope, i.e. α-1,3-galactosyl moieties decorating cell surface proteins, using the lectin IB4. IB4 binding was assayed either *in situ* or by FACS sorting.

For *in situ* binding, cells were fixed to solid supports with cold methanol for 5 mins, cells were rinsed in PBS (phosphate buffered saline) and non-specific IB4 binding was blocked with 1% BSA in PBS for 10 mins. Fixed cells were probed using 20 ug/ml IB4-biotin (Sigma) in 1% BSA, PBS for 30 mins at room temperature, cells were washed in PBS then probed with a 1:200 dilution of ExtrAvidin-FITC (Sigma) in PBS for 30 mins followed by further rinses in PBS. Cells were then examined using fluorescence microscopy, under these conditions the outer surface of PK2 control cells uniformly stained green.

For FACS analysis, cells were suspended after treatment with trypsin, washed in HBSS/Hepes (Hank's buffered saline solution with 20 mM Hepes, pH7.4) and probed with 10 ug/ml IB4-biotin (Sigma) in HBSS/Hepes for 45 mins at 4°C. Cells were washed in HBSS/Hepes, probed with a 1:200 dilution of ExtrAvidin-FITC (Sigma) in HBSS/Hepes for 45 mins at 4°C at and rinsed in cold HBSS/Hepes prior to FACS sorting.

Using this approach transformed cell lines are assayed for Galt inactivation and quantitative assessment of construct effectiveness is determined. Moreover cell lines showing Galt inactivation are isolated and subject to further molecular

analyses to determine the mechanism of gene inactivation.

EXAMPLE 8

Preparation of plasmid construct cassettes for use in achieving co-suppression

1. Generic RNA Isolation, cDNA synthesis and PCR protocol

Total RNA was purified from the indicated cell lines using an RNeasy Mini Kit according to the supplier's protocol (Qiagen). To prepare cDNA, this RNA was reverse-transcribed using Omniscript Reverse Transcriptase (Qiagen). Two micrograms of total RNA was reverse-transcribed using 1μM oligo dT (Sigma) as a primer in a 20 μl reaction according to the supplier's protocol (Qiagen).

To amplify specific products, 2 μl of this mixture was used as a substrate for PCR amplification, which was performed using HotStarTaq DNA polymerase according to the supplier's protocol (Qiagen). PCR amplification conditions involved an initial activation step at 95°C for 15 min, followed by 35 amplification cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 60 sec, with a final elongation step at 72°C for 4 min.

PCR products to be cloned were usually purified using a QIAquick PCR Purification Kit (Qiagen); in instances where multiple fragments were generated by PCR, the fragment of the correct size was purified from agarose gels using a QIAquick Gel Purification Kit (Qiagen) according to the supplier's protocol.

Amplification products were then cloned into pCR (registered trademark) 2.1-TOPO (Invitrogen) according to the supplier's protocol.

2. Generic cloning techniques

To prepare the constructs described below, insert fragments were excised from

intermediate vectors using restriction enzymes according to the supplier's protocols (Roche) and fragments purified from agarose gels using QIAquick Gel Purification Kits (Qiagen) according to the supplier's protocol. Vectors were usually prepared by restriction digestion and treated with Shrimp Alkaline Phosphatase according to the supplier's protocol (Amersham). Vector and inserts were ligated using T4 DNA ligase according to the supplier's protocols (Roche) and transformed into competent *Escherichia coli* strain DH5α using standard procedures (Sambrook, Fritsch et al. 1989).

3. Constructs

(a) Commercial plasmids

Plasmid pEGFP-N1

Plasmid pEGFP-N1 (Clontech) contains the CMV IE promoter operably connected to an open reading frame encoding a red-shifted variant of the wild-type GFP which has been optimized for brighter fluorescence. The specific GFP variant encoded by pEGFP-N1 has been disclosed by (Cormack, Valdivia et al. 1996). Plasmid pEGFP-N1 contains a multiple cloning site comprising *Bgl*II and *Bam*HI sites and many other restriction endonuclease cleavage sites, located between the CMV IE promoter and the EGFP open reading frame. The plasmid pEGFP-N1 will express the EGFP protein in mammalian cells. In addition, structural genes cloned into the multiple cloning site will be expressed as EGFP fusion polypeptides if they are in-frame with the EGFP-encoding sequence and lack a functional translation stop codon. The plasmid further comprises an SV40 polyadenylation signal downstream of the EGFP open reading frame to direct proper processing of the 3'-end of mRNA transcribed from the CMV IE promoter sequence (SV40 pA). The plasmid further comprises the SV40 origin of replication functional in animal cells; the neomycin-resistance gene comprising the SV40 E (early) promoter operably connected to the neomycin/kanamycin-resistance gene derived from Tn5 and the HSV thymidine kinase polyadenylation signal, for selection of transformed cells on kanamycin, neomycin or geneticin; the pUC19 origin of replication which is

functional in bacterial cells and the f1 origin of replication for single-stranded DNA production.

Plasmid pBluescript II SK+

Plasmid pBluescript II SK$^+$ (Stratagene) comprises the lacZ promoter sequence and lacZ-α transcription terminator, with multiple restriction endonuclease cloning sites located there between. Plasmid pBluescript II SK$^+$ is designed to clone nucleic acid fragments by virtue of the multiple restriction endonuclease cloning sites. The plasmid further comprises the ColEI and fl origins of replication and the ampicillin-resistance gene (α-lactamase).

Plasmid pCR (registered trademark) 2.1

Plasmid pCR (registered trademark) 2.1 (Invitrogen) is a T-tailed vector comprising the lacZ promoter sequence and lacZ-α transcription terminator, with a cloning site for the insertion of structural gene sequences there between. Plasmid pCR (registered trademark) 2.1 is designed to clone nucleic acid fragments by virtue of the A-overhang frequently synthesized by *Taq* polymerase during the polymerase chain reaction. The plasmid further comprises the ColEI and fl origins of replication and kanamycin-resistance and ampicillin-resistance genes.

Plasmid pCR (registered trademark) 2.1-TOPO

Plasmid pCR (registered trademark) 2.1-TOPO (Invitrogen) is a T-tailed vector comprising the lacZ promoter sequence and lacZ-α transcription terminator, with multiple restriction endonuclease cloning sites located there between. Plasmid pCR (registered trademark) 2.1-TOPO is provided with covalently bound topoisomerase I enzyme for fast cloning. The plasmid further comprises the ColEI and fl origins of replication and the kanamycin and ampicillin-resistance genes.

(b) Intermediate cassettes

Plasmid TOPO.BGI2

Plasmid TOPO.BGI2 comprises the human β-globin intron number 2 (BGI2) placed in the multiple cloning region of plasmid pCR (registered trademark) 2.1-TOPO. To produce this plasmid, the human β-globin intron number 2 (BGI2) was amplified from human genomic DNA using the amplification primers:

GD1 GAG CTC TTC AGG GTG AGT CTA TGG GAC CC [SEQ ID NO:17]

and

GA1 CTG CAG GAG CTG TGG GAG GAA GAT AAG AG [SEQ ID NO:18]

and cloned into plasmid pCR (registered trademark) 2.1-TOPO. BGI2 is a functional intron sequence that is capable of being post-transcriptionally cleaved from RNA transcripts containing it in mammalian cells.

(c) Plasmid cassettes

Plasmid pCMV.cass

Plasmid pCMV.cass is an expression cassette for driving expression of a structural gene sequence under control of the CMV-IE promoter sequence. Plasmid pCMV.cass was derived from pEGFP-N1 by deletion of the EGFP open reading frame as follows: Plasmid pEGFP-N1 was digested with *Pin*AI and *Not*I, blunt-ended using *Pfu*I DNA polymerase and then religated. Structural gene sequences are cloned into pCMV.cass using the multiple cloning site, which is identical to the multiple cloning site of pEGFP-N1, except it lacks the *Pin*AI site.

Plasmid pCMV.BGI2.cass

To create pCMV.BGI2.cass, the human β-globin intron 2 sequence was isolated

as a *SacI*/*PstI* fragment from TOPO.BGI2 and cloned between the *SacI* and *PstI* sites of pCMV.cass. In pCMV.BGI2.cass, any RNAs transcribed from the CMV promoter will include the human β-globin intron 2 sequences; these intron sequences will presumably be excised from transcripts as part of the normal intron processing machinery, since the intron sequences include both the splice donor and splice acceptor sequences necessary for normal intron processing.

EXAMPLE 9

Co-suppression of Green Fluorescent Protein in Porcine Kidney Type 1 cells in vitro

1. Culturing of cell lines

PK-1 cells (derived from porcine kidney epithelial cells) were grown as adherent monolayers using DMEM supplemented with 10% v/v FBS.

2. Preparation of genetic constructs

(a) Interim plasmids

Plasmid pBluescript.EGFP

Plasmid pBluescript.EGFP comprises the EGFP open reading frame derived from plasmid pEGFP-N1 placed in the multiple cloning region of plasmid pBluescript II SK$^+$ (Stratagene). To produce this plasmid, the EGFP open reading frame was excised from pEGFP-N1 by restriction endonuclease digestion using the enzymes *NotI* and *XhoI* and ligated into *NotI*/*XhoI*-digested pBluescript II SK$^+$.

(b) Test plasmids

Plasmid pCMV.EGFP

Plasmid pCMV.EGFP is capable of expressing the entire EGFP open reading

frame under the control of CMV-IE promoter sequence. To produce pCMV.EGFP, the EGFP sequence from pBluescript.EGFP was sub-cloned in the sense orientation as a *Bam*HI-to-*Sac*I fragment into *Bgl*II/*Sac*I-digested pCMV.cass.

3. Detection of co-suppression phenotype

(a) Insertion of EGFP-expressing transgene into PK-1 cells

Transformations were performed in 6-well tissue culture vessels (Nunc). Individual wells were seeded with 4 x 10^4 PK-1 cells in 2 ml of DMEM, 10% v/v FBS and incubated at 37°C in 5% v/v CO_2 until the monolayer was 60–90% confluent, typically 16 to 24 hr.

To transform a single plate (six wells), 12 μg of pCMV.EGFP plasmid DNA and 108 μl of GenePORTER2 (trademark) (Gene Therapy Systems) were diluted into OPTI-MEM I (registered trademark) medium (Life Technologies) to obtain a final volume of 6 ml and incubated at room temperature for 45 min.

The tissue growth medium was removed from each well and the monolayer therein was washed with 1 ml of 1 x PBS (Sigma). The monolayers were overlayed with 1 ml of the plasmid DNA/GenePORTER2 (trademark) conjugate for each well and incubated at 37°C in 5% v/v CO_2 for 4.5 hr.

OPTI-MEM-I (registered trademark) (1 ml) supplemented with 20% v/v FBS was added to each well and the vessel incubated for a further 24 hr, at which time the monolayers were washed with 1 x PBS and medium was replaced with 2 ml of fresh DMEM including 10% v/v FBS. Cells transformed with pCMV.EGFP were examined after 24–48 hr for transient EGFP expression using fluorescence microscopy at a wavelength of 500–550 nm.

Forty-eight hr after transfection the medium was removed, the cell monolayer washed with 1 x PBS and 4 ml of fresh DMEM containing 10% v/v FBS supplemented with 1.5 mg/ml genetecin (Life Technologies) was added to each

well. Genetecin was included in the medium to select for stably transformed cell lines. The DMEM, 10% v/v FBS, 1.5 mg/ml genetecin medium was changed every 48–72 hr. After 21 days of selection, stable, EGFP-expressing PK-1 colonies were apparent.

Individual colonies of stably transfected PK-1 cells were cloned, maintained and stored as described in Example 5, above.

A number of parental cell lines were transformed with pCMV.EGFP. Following continuous culture, in many of these lines GFP expression was either extremely low or completely undetectable as listed in Table 3 and shown in Figure 42.

TABLE 3

Parental Cell line	Number of cloned lines examined	Number of cell lines with extremely low or undetectable GFP
PK-1 (pig)	59	2
MM96L (human)	12	4
B16 (mouse)	12	10
MDAMB468 (human)	11	1

These data demonstrate that silencing of GFP expression occurred frequently in different types of cell lines, established from three different species.

4. Southern analysis

Individual transgenic PK-1 cell lines (transfected and co-transfected) are analyzed by Southern blot analysis to confirm integration and determine copy number of the transgenes. The procedure is carried out according to the protocol set forth in Example 1, above. An example is illustrated in Figure 70.

EXAMPLE 10

Co-suppression of Bovine Enterovirus in Madin Darby Bovine Kidney Type CRIB-1 cells In vitro

1. Culturing of cell lines

CRIB-1 cells (derived from bovine kidney epithelial cells) were grown as adherent monolayers using DMEM supplemented with 10% v/v Donor Calf Serum (DCS; Life Technologies), as described in Example 1.

2. Preparation of genetic constructs

(a) Interim plasmid

Plasmid pCR.BEV2

The complete Bovine enterovirus (BEV) RNA polymerase coding region was amplified from a full-length cDNA clone encoding same, using primers:

BEV-1 CGG CAG ATC CTA ACA ATG GCA GGA CAA ATC GAG TAC ATC [SEQ ID NO:19]

and

BEV-3 GGG CGG ATC CTT AGA AAG AAT CGT ACC AC [SEQ ID NO:20].

Primer BEV-1 comprises a *BglII* restriction endonuclease site at positions 4–9, inclusive, and an ATG start site at positions 16–18, inclusive. Primer BEV-3 comprises a *Bam*HI restriction enzyme site at positions 5–10, inclusive, and the complement of a TAA translation stop signal at positions 11–13, inclusive. As a consequence, an open reading frame comprising a translation start signal and a translation stop signal is contained between the *Bgl*I and *Bam*HI restriction sites. The amplified fragment was cloned into pCR2.1 to produce plasmid pCR.BEV2.

Plasmid pBS.PFGE

Plasmid pBS.PFGE contains the EGFP coding sequences from pEGFP-N1 cloned in antisense orientation into the polylinker of pBluescript II SK$^+$. To generate this plasmid, the EGFP coding sequences from pEGFP-N1 was cloned as a *Not*I-to-*Sac*I fragment into *Not*I/*Sac*I-digested pBluescript II SK$^+$.

(b) Test plasmids

Plasmid pCMV.BEV2.BGI2.2VEB

Plasmid pCMV.BEV2.BGI2.2VEB contains an inverted repeat or palindrome of the BEV polymerase coding region that is interrupted by the insertion of the human β-globin intron 2 sequence therein. Plasmid pCMV.BEV2.BGI2.2VEB was constructed in successive steps: (i) the BEV2 sequence from plasmid pCR.BEV2 was sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bgl*II-digested pCMV.BGI2.cass to make plasmid pCMV.BEV2.BGI2, and (ii) the BEV2 sequence from plasmid pCR.BEV2 was sub-cloned in the antisense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.BEV2.BGI2 to make plasmid pCMV.BEV2.BGI2.2VEB.

Plasmid pCMV.BEV.EGFP.VEB

Plasmid pCMV.BEV.EGFP.VEB contains an inverted repeat or palindrome of the BEV polymerase coding region that is interupted by EGFP coding sequences which act as a stuffer fragment. To generate this plasmid, the EGFP coding sequence from pBS.PFGE was isolated as an *Eco*RI fragment and cloned into *Eco*RI-digested pCMV.cass in the sense orientation relative to the CMV promoter to generate pCMV.EGFP.cass. Plasmid pCMV.BEV.EGFP.VEB was constructed in successive steps: (i) the BEV polymerase sequence from plasmid pCR.BEV2 was sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bgl*II-digested pCMV.EGFP.cass to make plasmid pCMV.BEV.EGFP, and (ii) the BEV polymerase sequence from plasmid pCR.BEV2 was sub-cloned in the antisense

orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.BEV.EGFP to make plasmid pCMV. BEV.EGFP.VEB.

3. Detection of co-suppression phenotype

(a) Insertion of Bovine enterovirus RNA polymerase-expressing transgene into CRIB-1 cells

Transformations were performed in 6-well tissue culture vessels. Individual wells were seeded with 2 x 10^5 CRIB-1 cells in 2 ml of DMEM, 10% v/v DCS and incubated at 37°C in 5% v/v CO_2 until the monolayer was 60–90% confluent, typically 16–24 hr.

The following solutions were prepared in 10 ml sterile tubes:

Solution A: For each transfection, 1 µg of DNA (pCMV.BEV2.BGI2.2VEB or pCMV.EGFP) was diluted into 100 µl of OPTI-MEM-I (registered trademark) and;

Solution B: For each transfection, 10 µl of LIPOFECTAMINE (trademark) Reagent (Life Technologies) was diluted into 100 µl OPTI-MEM-I (registered trademark).

The two solutions were combined and mixed gently, and incubated at room temperature for 45 min to allow DNA-liposome complexes to form. While complexes formed, the CRIB-1 cells were rinsed once with 2 ml of OPTI-MEM I (registered trademark).

For each transfection, 0.8 ml of OPTI-MEM I (registered trademark) was added to the tube containing the complexes, the tube mixed gently, and the diluted complex solution overlaid onto the rinsed CRIB-1 cells. Cells were then incubated with the complexes at 37°C in 5% v/v CO_2 for 16–24 hr.

Transfection mixture was then removed and the CRIB-1 monolayers overlaid with 2 ml of DMEM, 10% v/v DCS. Cells were incubated at 37°C in 5% v/v CO_2 for approximately 48 hr. To select for stable transformants, the medium was replaced every 72 hr with 4 ml of DMEM, 10% v/v DCS, 0.6 mg/ml geneticin.

Cells transformed with the transfection control pCMV.EGFP were examined after 24–48 hr for transient EGFP expression using fluorescence microscopy at a wavelength of 500–550 nm. After 21 days of selection, stably transformed CRIB-1 colonies were apparent.

Individual colonies of stably transfected CRIB-1 cells were cloned, maintained and stored as described in Example 1.

(b) Determination of Bovine Enterovirus titre

The BEV isolate used in these experiments was a cloned isolate, K2577. To amplify BEV virus from this stock, cells were infected with 5 μl of viral stock per well and the virus allowed to replicate for 48 hr, as described below. Culture medium was harvested at this time and transferred to a screw-capped tube. Dead cells and debris were removed by centrifugation at 3,500 rpm for 15 min at 4°C in a Sigma 3K18 centrifuge. The supernatant was decanted into a fresh tube and centrifuged at 20,000 rpm for 30 min at 4°C in a Beckman J2-M1 centrifuge to remove remaining debris. The supernatant was decanted and this new BEV stock titred as described below and stored at 4°C.

Absolute:

In a 6-well tissue culture plate, 2.5 x 10^5 CRIB-1 cells were seeded per well in 2 ml DMEM, 10% v/v DCS. The cells were incubated at 37°C in 5% v/v CO_2 until 90–100% confluent.

BEV was diluted in serum-free DMEM at dilutions of 10^{-1} to 10^{-9}. Medium was aspirated from the CRIB-1 monolayers and the cells overlaid with 2 ml of 1 x PBS

and the vessels rocked gently to wash the monolayer. PBS was aspirated from the monolayer and the wash repeated.

One ml of diluted virus solutions (10^{-4} to 10^{-9}) was added directly onto the rinsed CRIB-1 cells, using one dilution per well in duplicate. The cells were incubated with BEV for 1 hr at 37°C in 5% v/v CO_2 with gentle agitation. Medium was aspirated and the infected cells overlaid with 3 ml of nutrient agar (1% Noble Agar in DMEM).

The agar overlay was allowed to set and the plates incubated (inverted) at 37°C in 5% v/v CO_2 for 18–24 hr. Following incubation, each well was overlaid with 3 ml of Neutral Red Agar (1.7 ml Neutral Red Solution (Life Technologies) in 100 ml Nutrient Agar). The overlay was allowed to set and the plates incubated (inverted) in the dark at 37°C in 5% v/v CO_2 for 18–24 hr. Plaques were counted to determine the titre of the BEV viral stock.

Empirical:

In a 24-well tissue culture plate, 4×10^4 CRIB-1 cells were seeded per well in 800 μl DMEM, 10% v/v DCS. The cells were incubated at 37°C in 5% v/v CO_2 until 90–100% confluent.

From concentrated BEV viral stock, BEV was diluted in serum-free DMEM at dilutions of 10^{-1} to 10^{-9}. The medium was aspirated from the CRIB-1 monolayers and the monolayers overlaid with 800 μl of 1 x PBS and washed by gently rocking the tissue culture vessel. PBS was aspirated from the monolayers and the wash repeated.

200 μl of the diluted virus solutions (10^{-3} to 10^{-9}) was added immediately directly onto the rinsed CRIB-1 cells using one dilution per well in duplicate. The CRIB-1 cells were incubated with BEV for 24 hr at 37°C in 5% v/v CO_2 and each well inspected microscopically for cell lysis. A further 600 μl of serum-free DMEM was then added to each well. After a further 24 hr, each well was inspected

microscopically for cell lysis. The working dilution is the minimum viral concentration that kills most of the CRIB-1 cells after 24 hr and all cells after 48 hr.

(c) Bovine enterovirus challenge of CRIB-1 cells transformed with pCMV.BEV2.BGI2.2VEB

In a 24-well tissue culture plate, 4 x 10^4 CRIB-1 cells per well were seeded in triplicate, in 800 μl DMEM, 10% v/v DCS. The cells were incubated at 37°C in 5% v/v CO_2 until 90–100% confluent.

From concentrated BEV viral stock, BEV virus was diluted in serum-free DMEM at an appropriate dilution. In addition, the BEV viral stock was diluted to 10x and 0.1x the working dilution (typically 10^{-4} to 10^{-6} pfu).

Medium was aspirated from the CRIB-1 monolayers and the monolayers overlaid with 800 μl of 1 x PBS and washed gently by rocking the tissue culture vessel. PBS was aspirated from the monolayers and the wash repeated.

200 μl of the diluted virus solutions (one dilution per replicate) was added immediately, directly onto the rinsed CRIB-1 cells. The cells were incubated with BEV for 24 hr at 37°C in 5% v/v CO_2, and each well inspected microscopically for cell lysis. A further 600 μl of serum-free DMEM was added to each well. After a further 24 hr, each well was inspected microscopically for cell lysis.

(d) Generation of CRIB-1 viral tolerant cell lines

To determine whether cells transformed with pCMV.BEV.EGFP.VEB or pCMV.BEV2.BGI2.2VEB became tolerant to BEV infection, transformed cell lines were challenged with dilutions of BEV and monitored for survival. To overcome inherent variation in these assays, multiple challenges were performed and lines consistently showing viral tolerance were isolated for further examination. Results of these experiments are shown below in Tables 4 and 5.

TABLE 4

CRIB-1 cells transfected with pCMV.BEV.EGFP.VEB (CRIB-1 EGFP)

Cell line	Challenge 1		Challenge 2		Challenge 3		Challenge 4	
	10^4	10^5	10^4	10^5	10^4	10^5	10^4	10^5
CRIB-1	nd	nd	–	–	–	–	–	–
CRIB-1 EGFP # 1	–	–	–	–	–	–	+	–
CRIB-1 EGFP # 3	–	–	+	++	–	–	nd	nd
CRIB-1 EGFP # 4	–	–	–	–	–	–	++	–
CRIB-1 EGFP # 5	–	–	+	+++	–	–	nd	nd
CRIB-1 EGFP # 6	–	+	–	–	–	–	–	–
CRIB-1 EGFP # 7	+	+	–	+	+	+	nd	nd
CRIB-1 EGFP # 8	+	+++	+	+	+	+++	–	++
CRIB-1 EGFP # 9	–	–	–	+	+	+	nd	nd
CRIB-1 EGFP # 10	–	+	–	+	+	++	nd	nd
CRIB-1 EGFP # 11	+	++	–	–	+	+++	nd	nd
CRIB-1 EGFP # 12	–	+	+	++	+	+	nd	nd

	Challenge 1		Challenge 2		Challenge 3		Challenge 4	
CRIB-1 EGFP # 13	–	–	+	+	–	–	nd	nd
CRIB-1 EGFP # 14	++	++	+	++	++	+	+	+
CRIB-1 EGFP # 15	–	+	++	++	+	++	nd	nd
CRIB-1 EGFP # 16	–	+	–	++	+	++	nd	nd
CRIB-1 EGFP # 17	–	–	+	+	–	–	nd	nd
CRIB-1 EGFP # 18	+	+	++	+	++	++	nd	nd
CRIB-1 EGFP # 20	–	–	–	–	+	+++	nd	nd
CRIB-1 EGFP # 21	–	++	+	++	+	+	nd	nd
CRIB-1 EGFP # 22	–	+	+	+	+	+	nd	nd
CRIB-1 EGFP # 23	–	–	–	+++	–	++	–	–
CRIB-1 EGFP # 24	–	–	+	++	–	+		
'CRIB-1 EGFP # 25	–	+	–	+++	–	–	nd	nd
CRIB-1 EGFP # 26	+	++	++	+++	++	+++	–	–

– no cells surviving

\+ 1–10% of cells surviving.

++ 10–90% of cells surviving.

+++ 90%+ of cells surviving

nd not done.

TABLE 5

CRIB-1 cells transfected with pCMV.BEV2.BGI2.2VEB (CRIB-1 BGI2)

Cell line	Challenge 1		Challenge 2		Challenge 3		Challenge 4	
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
CRIB-1	nd	nd	–	–	–	–	–	–
CRIB-1 BGI2 # 1	–	–	–	–	–	–	nd	nd
CRIB-1 BGI2 # 2	–	–	–	+	–	–	–	–
CRIB-1 BGI2 # 3	–	–	++	++	+	++	nd	nd
CRIB-1 BGI2 # 4	–	–	–	+	–	–	nd	nd
CRIB-1 BGI2 # 5	–	–	–	++	–	–	nd	nd
CRIB-1 BGI2 # 6	+	+	+++	++	+	+	nd	nd
CRIB-1 BGI2 # 7	+	+	–	+++	–	–	nd	nd
CRIB-1 BGI2 # 8	–	+	+++	++	–	+	nd	nd
CRIB-1 BGI2 # 9	–	+	–	++	+	++	–	++
CRIB-1 BGI2 # 10	++	++	++	+++	+	+	–	–
CRIB-1 BGI2 # 11	+	++	+	+	–	+	nd	nd
CRIB-1 BGI2 # 12	+	+	+	+++	–	–	nd	nd

	Challenge 1		Challenge 2		Challenge 3		Challenge 4	
CRIB-1 BGI2 # 13	–	–	+++	+++	–	–	nd	nd
CRIB-1 BGI2 # 14	+	++	+	++	+	+	nd	nd
CRIB-1 BGI2 # 15	+	+	+	++	+	++	–	–
CRIB-1 BGI2 # 16	–	–	–	–	–	–	nd	nd
CRIB-1 BGI2 # 17	–	+	–	++	–	–	nd	nd
CRIB-1 BGI2 # 18	–	–	–	+++	–	–	nd	nd
CRIB-1 BGI2 # 19	–	–	–	++	+	+++	+	+++
CRIB-1 BGI2 # 20	+	+	+	+++	+	+	nd	nd
CRIB-1 BGI2 # 21	–	–	–	–	–	–	–	–
CRIB-1 BGI2 # 22	–	–	–	–	–	–	–	–
CRIB-1 BGI2 # 23	–	+	+++	+++	+	+	nd	nd
CRIB-1 BGI2 # 24	–	++	+++	+	–	–	nd	nd

- no cells surviving

+ 1–10% of cells surviving.

++ 10–90% of cells surviving.

+++ 90%+ of cells surviving

nd not done.

These data showed that viral-tolerant cell lines could be defined in this fashion. In addition, cells which survived this viral challenge could be grown up for further analyses.

To further define the degree of viral tolerance in such cell lines, the cell line CRIB-1 BGI2 #19, and viral-tolerant cells grown from cells that survived the initial challenge (line CRIB-1 BGI2 #19(tol)), were further analyzed using finer scale (3-fold) serial dilutions of BEV in triplicate. The results of these experiments are shown in Table 6.

TABLE 6

Cell line	Dilution of viral stock					
	3.3×10^{-4}	1.1×10^{-4}	3.7×10^{-5}	1.2×10^{-5}	4.1×10^{-6}	1.3×10^{-6}
CRIB-1 Replicate 1	–	–	–	–	–	+++
CRIB-1 Replicate 1	–	–	–	–	–	+
CRIB-1 Replicate 1	–	–	–	–	–	+++
CRIB-1 BGI2 #19 Replicate 1	–	–	+	+	++	+++
CRIB-1 BGI2 #19 Replicate 2	–	–	–	–	++	+++
CRIB-1 BGI2 #19 Replicate 3	–	–	–	+	+++	+++
CRIB-1 BGI2 #19(tol) Replicate 1	–	–	+	+	+++	+++
CRIB-1 BGI2 #19(tol) Replicate 2	–	–	+	+	++	+++
CRIB-1 BGI2 #19(tol) Replicate 3	–	–	+	+	+++	+++

– no cells surviving 48 hr post-infection

\+ 1–10% of cells surviving 48 hr post-infection.

++ 10–90% of cells surviving 48 hr post-infection.

+++ 90%+ of cells surviving 48 hr post-infection.

These data showed that the cell lines CRIB-1 BGI2 #19 and CRIB-1 BGI2 #19(tol) were tolerant to higher titres of BEV than the parental CRIB-1 line. Figure 44 shows micrographs comparing CRIB-1 and CRIB-1 BGI2 #19(tol) cells before and 48 hr after BEV infection.

EXAMPLE 11

Co-suppression of Tyrosinase in Murine Type B16 cells In vitro

1. Culturing of cell lines

B16 cells derived from murine melanoma (ATCC CRL-6322) were grown as adherent monolayers in RPMI 1640 supplemented with 10% v/v FBS, as described in Example 1.

2. Preparation of genetic constructs

(a) Interim plasmid

Plasmid TOPO.TYR

Total RNA was purified from cultured murine B16 melanoma cells and cDNA prepared as described in Example 8.

To amplify a region of the murine tyrosinase gene, 2 μl of this mixture was used as a substrate for PCR amplification using the primers:

TYR-F: GTT TCC AGA TCT CTG ATG GC [SEQ ID NO:21]

and

TYR-R: AGT CCA CTC TGG ATC CTA GG [SEQ ID NO:22].

The PCR amplification was performed using HotStarTaq DNA polymerase according to the supplier's protocol (Qiagen). PCR amplification conditions involved an initial activation step at 95°C for 15 mins, followed by 35 amplification cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec, with a final elongation step at 72°C for 4 min.

The PCR amplified region of tyrosinase was column-purified (PCR purification column, Qiagen) and then cloned into pCR (registered trademark) 2.1-TOPO according to the supplier's instructions (Invitrogen) to make plasmid TOPO.TYR.

(b) Test plasmids

Plasmid pCMV.TYR.BGI2.RYT

Plasmid pCMV.TYR.BGI2.RYT contains an inverted repeat, or palindrome, of a region of the murine tyrosinase gene that is interrupted by the insertion of the human β-globin intron 2 (BGI2) sequence therein. Plasmid pCMV.TYR.BGI2.RYT was constructed in successive steps: (i) the TYR sequence from plasmid TOPO.TYR was sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bgl*II-digested pCMV.BGI2 to make plasmid pCMV.TYR.BGI2, and (ii) the TYR sequence from plasmid TOPO.TYR was sub-cloned in the antisense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.TYR.BGI2 to make plasmid pCMV.TYR.BGI2.RYT.

Plasmid pCMV.TYR

Plasmid pCMV.TYR contains a single copy of mouse tyrosinase cDNA sequence, expression of which is driven by the CMV promoter. Plasmid pCMV.TYR was constructed by cloning the TYR sequence from plasmid TOPO.TYR as a *Bam*HI-

to-*Bgl*II fragment into *Bam*HI-digested pCMV.cass and selecting plasmids containing the TYR sequence in a sense orientation relative to the CMV promoter.

Plasmid pCMV.TYR.TYR

Plasmid pCMV.TYR.TYR contains a direct repeat of the mouse tyrosinase cDNA sequence, expression of which is driven by the CMV promoter. Plasmid pCMV.TYR.TYR was constructed by cloning the TYR sequence from plasmid TOPO.TYR as a *Bam*HI-to-*Bgl*II fragment into *Bam*HI-digested pCMV.TYR and selecting plasmids containing the second TYR sequence in a sense orientation relative to the CMV promoter.

3. Detection of co-suppression phenotype

(a) Reduction of melanin pigmentation through PTGS of tyrosinase by insertion of a region of the tyrosinase gene into murine melanoma B16 cells

Tyrosinase is the major enzyme controlling pigmentation in mammals. If the gene is inactivated, melanin will no longer be produced by the pigmented B16 melanoma cells. This is essentially the same process that occurs in albino animals.

Transformations were performed in 6-well tissue culture vessels. Individual wells were seeded with 1 x 10^5 cells in 2 ml of RPMI 1640, 10% v/v FBS and incubated at 37°C in 5% v/v CO_2 until the monolayer was 60–90% confluent, typically 16–24 hr.

Subsequent procedures were as described above in Example 8, except that B16 cells were incubated with the DNA-liposome complexes at 37°C in 5% v/v CO_2 for 3–4 hr only.

Individual colonies of stably transfected B16 cells were cloned, maintained and stored as described in Example 1.

Thirty six clones stably transformed with pCMV.TYR.BGI2.RYT, 34 clones stably transformed with pCMV.TYR and 37 clones stably transformed with pCMV.TYR.TYR were selected for subsequent analyses.

When the endogenous tyrosinase gene is post-transcriptionally silenced, melanin production in the B16 cells is reduced. B16 cells that would normally appear to contain a dark brown pigment will now appear lightly pigmented or unpigmented.

(b) Visual monitoring of melanin production in transformed B16 cell lines

To monitor melanin content of transformed cell lines, cells were trypsinized and transferred to media containing FBS to inhibit trypsin activity. Cells were then counted with a haemocytometer and 2×10^6 cells transferred to a microfuge tube. Cells were collected by centrifugation at 2,500 rpm for 3 min at room temperature and pellets examined visually.

Five clones transformed with pCMV.TYR.BGI2.RYT, namely B16.2 1.11, B16 3.1.4, B16 3.1.15, B16 4.12.2 and B16 4.12.3, were considerably paler than the B16 controls (Figure 45). Four clones transformed with pCMV.TYR (B16+Tyr 2.3, B16+Tyr 2.9, B16+Tyr 3.3, B16+Tyr 3.7 and B16+Tyr 4.10) and five clones transformed with pCMV.TYR.TYR (B16+TyrTyr 1.1, B16+TyrTyr 2.9, B16+TyrTyr 3.7, B16+TyrTyr 3.13 and B16+TyrTyr 4.4) were also significantly paler than the B16 controls.

(c) Identification of melanin by staining according to Schmorl

Specific diagnosis for the presence of cellular melanin can be achieved using a modified Schmorl's melanin staining (Koss 1979). Using this method, the presence of melanin in the cell is detected by a specific staining procedure that converts melanin to a greenish-black pigment.

Cell populations to be stained were resuspended at a concentration of 500,000 cells per ml in RPMI 1640 medium. Volumes of 200 μl were dropped onto surface-

sterilized microscope slides and slides were incubated at 37°C in a humidified atmosphere in 100 mm TC dishes until cells had adhered firmly. The medium was removed and cells were fixed by air drying on a heating block at 37°C for 30 min then post-fixed with 4% w/v paraformaldehyde (Sigma) in PBS for 1 hr. Fixed cells were hydrated by dipping in 96% v/v ethanol in distilled water, 70% v/v ethanol, 50% v/v ethanol then distilled water. Slides with adherent cells were left for 1 hr in a ferrous sulfate solution (2.5% w/v ferrous sulfate in water) then rinsed in four changes of distilled water, 1 min each. Slides were left for 30 min in a solution of potassium ferricyanide (1% w/v potassium ferricyanide in 10% v/v acetic acid in distilled water). Slides were dipped in 1% v/v acetic acid (15 dips) then dipped in distilled water (15 dips).

Cells were stained for 1–2 min in a Nuclear Fast Red preparation (0.1% w/v Nuclear Fast Red (C.I. 60760 Sigma N 8002) dissolved with heating in 5% w/v ammonium sulfate in water). Fixed and stained cells on slides were washed by dipping in distilled water (15 dips). Cover slips were mounted on slides in glycerol/DABCO (25 mg/ml DABCO (1,4-diazabicyclo(2.2.2)octane (Sigma D 2522)) in 80% v/v glycerol in PBS). Cells were examined by bright field microscopy using a 100x oil immersion objective.

The results of staining with Schmorl's stain correlated with the simple visual data illustrated in Figure 45 for all cell lines. When B16 cells were stained with the above procedure, melanin was obvious in most cells. In contrast, fewer cells stained for melanin in the transformed lines B16 2.1.11, B16 3.1.4, B16 3.1.15, B16 4.12.2, B16 4.12.3, B16 Tyr 2.3, B16 Tyr 2.9, B16 Tyr 4.10, B16 TyrTyr 1.1, B16 TyrTyr 2.9 and B16 TyrTyr 3.7, consistent with the reduced gross pigmentation observed in these cell lines.

(d) Assaying tyrosinase enzyme activity in transformed cell lines

Tyrosinase catalyzes the first two steps of melanin synthesis: the hydroxylation of tyrosine to dopa (dihydroxyphenylalanine) and the oxidation of dopa to dopaquinone. Tyrosinase can be measured as its dopa oxidase activity. This

assay uses Besthorn's hydrazone (3-methyl-2-benzothiazolinonehydrazone hydrochloride, MBTH) to trap dopaquinone formed by the oxidation of L-dopa. Presence of a low concentration of N,N'-dimethylformamide in the assay mixture renders the MBTH soluble and the method can be used over a range of pH values. MBTH reacts with dopaquinone by a Michael addition reaction and forms a dark pink product whose presence is monitored using a spectrophotometer or plate reader. It is assumed that the reaction of the MBTH with dopaquinone is very rapid relative to the enzyme-catalyzed oxidation of L-dopa. The rate of production of the pink pigment can be used as a quantitative measure of enzyme (Winder and Harris 1991; Dutkiewicz, Albert et al. 2000).

B16 cells and transformed B16 cell lines were plated into individual wells of a 96-well plate in triplicate. Constant numbers of cells (25,000) were transferred into individual wells and cells were incubated overnight. Tyrosinase assays were performed as described below after either 24 or 48 hr incubation.

Individual wells were washed with 200 μl PBS and 20 μl of 0.5% v/v Triton X-100 in 50mM sodium phosphate buffer (pH 6.9) was added to each well. Cell lysis and solubilisation was achieved by freeze-thawing plates at -70°C for 30 min, followed by incubating at room temperature for 25 min and 37°C for 5 min.

Tyrosinase activity was assayed by adding 190 μl freshly-prepared assay buffer (6.3mM MBTH, 1.1mM L-dopa, 4% v/v N,N'-dimethylformamide in 48mM sodium phosphate buffer (pH 7.1)) to each well. Colour formation was monitored at 505 nm in a Tecan plate reader and data collected using X/Scan Software. Readings were taken at constant time intervals and reactions monitored at room temperature, typically 22°C. Results were calculated as the average of enzyme activities as measured for the triplicate samples. Data were analyzed and tyrosinase activity estimated at early time-points when product formation was linear, typically between 2 and 12 min. Results from these experiments are shown below in Tables 7 and 8.

TABLE 7

Cell Line	Tyrosinase activity (Δ OD 505 nm/min / 25 000 cells)	Relative tyrosinase activity compared with B16 cells (%)
B16	0.0123	100
B16 2.1.6 (Tyr.BGl2.ryT)	0.0108	87.8
B16 2.1.11(Tyr.BGl2.ryT)	0.0007	5.7
B16 3.1.4 (Tyr.BGl2.ryT)	0.0033	26.8
B16 3.1.15(Tyr.BGl2.ryT)	0.0011	8.9
B16 4.12.2(Tyr.BGl2.ryT)	0.0013	10.6
B16 4.12.3(Tyr.BGl2.ryT)	0.0011	8.9
B16 Tyr Tyr 1.1	0.0043	34
B16 Tyr Tyr 2.9	0.0042	34.1
B16 Tyr Tyr 3.7	0.0087	70.7

TABLE 8

Cell line	Tyrosinase activity (Δ OD 505 nm/min/ 25,000 cells)	Relative tyrosinase activity compared with B16 cells (%)
B16	0.0200	100
B16 Tyr 2.3	0.0036	18.2
B16 Tyr 2.9	0.0017	8.7
B16 Tyr 4.10	0.0034	17.2

These data showed that tyrosinase enzyme activity was reduced in lines transformed with the constructs pCMV.TYR.BGI2.RYT, pCMV.TYR and pCMV.TYR.TYR

4. Southern analysis

Individual transgenic B16 cell lines were analyzed by Southern blot analysis to confirm integration of the transgene, according to the protocol set forth in Example 1.

EXAMPLE 12

Co-suppression of HER-2 in MDA-MB-468 cells in vitro

HER-2 (also designated *neu* and *erbB-2*) encodes a 185 kDa transmembrane receptor tyrosine kinase that is constitutively activated at low levels and displays potent oncogenic activity when over-expressed. HER-2 protein over-expression occurs in about 30% of invasive human breast cancers. The biological function of HER-2 is not well understood. It shares a common structural organisation with other members of the epidermal growth factor receptor family and may participate in similar signal transduction pathways leading to changes in cytoskeleton

reorganisation, cell motility, protease expression and cell adhesion. Over-expression of *HER-2* in breast cancer cells leads to increased tumorigenicity, invasiveness and metastatic potential (Slamon, Clark et al. 1987).

1. Culturing of cell lines

Human MDA-MB-468 cells were cultured in RPMI 1640 supplemented with 10% v/v FBS. Cells were passaged twice a week by treating with trypsin to release cells and transferring a proportion of the culture to fresh medium, as described in Example 1.

2. Preparation of genetic constructs

(a) Interim Plasmid

Plasmid TOPO.HER-2

A region of the human *HER*-2 gene was amplified by PCR using human cDNA as a template. The cDNA was prepared from total RNA isolated from a human breast tumour line, SK-BR-3. Total RNA was purified as described in Example 8. Human *HER*-2 sequences were amplified using the primers:-

H1: CTC GAG AAG TGT GCA CCG GCA CAG ACA TG [SEQ ID NO:23]

and

H3: GTC GAC TGT GTT CCA TCC TCT GCT GTC AC [SEQ ID NO:24].

The amplification product was cloned into pCR (registered trademark) 2.1-TOPO to create the intermediate clone TOPO.HER-2.

(b) Test Plasmid

Plasmid pCMV.HER2.BGI2.2REH

Plasmid pCMV.HER2.BGI2.2REH contains an inverted repeat or palindrome of the HER-2 coding region that is interrupted by the insertion of the human β-globin intron 2 (BGI2) sequence therein. Plasmid pCMV.HER2.BGI2.2REH was constructed in successive steps: (i) the HER-2 sequence from plasmid TOPO.HER2 was sub-cloned in the sense orientation as a *SalI/XhoI* fragment into *SalI*-digested pCMV.BGI2.cass (Example 6) to make plasmid pCMV.HER2.BGI2, and (ii) the HER2 sequence from plasmid TOPO.HER2 was sub-cloned in the antisense orientation as a *SalI/XhoI* fragment into *XhoI*-digested pCMV.HER2.BGI2 to make plasmid pCMV.HER2.BGI2.2REH.

3. Determination of onset of co-suppression

(a) Transfection of HER-2 constructs

Transformations were performed in 6-well tissue culture vessels. Individual wells were seeded with 4 x 10^5 MDA-MB-468 cells in 2 ml of RPMI 1640 medium, 10% v/v FBS and incubated at 37°C in 5% v/v CO_2 until the monolayer was 60–90% confluent, typically 16–24 hr.

Subsequent procedures were as described above in Example 10, except that MDA-MB-468 cells were incubated with the DNA-liposome complexes at 37°C in 5% v/v CO_2 for 3–4 hr only. Thirty-six transformed cell lines were isolated for subsequent analysis.

(b) Post-transcriptional silencing of HER-2 in MDA-MB-468 cells

MDA-MB-468 cells over-express *HER-2* and PTGS of the gene in geneticin-selected clones derived from this cell line were tested by immunofluorescence labelling of clones (see Example 1) with a primary murine monoclonal antibody

directed against the extracellular domain of HER-2 protein. The primary antibody was a mouse Anti-erbB2 monoclonal antibody (Transduction Laboratories, Cat. No. E19420, an IgG2b isotype) used at 1/400 dilution; the secondary antibody was Alexa Fluor 488 goat anti-mouse IgG (H+L) conjugate (Molecular Probes, Cat. No. A-11001) used at 1/100 dilution. As a negative control, MDA-MB-468 cells (parental and transformed lines) were probed with Alexa Fluor 488 goat anti-mouse IgG (H+L) conjugate only.

Several MDA-MB-468 cell lines transformed with pCMV.HER2.BGI2.2REH were found to have reduced immunofluorescence, examples of which are illustrated in Figure 46.

(c) FACS analysis to define cell lines showing reduced expression of Her-2

To determine the level of expression of HER-2 in transformed cell lines, approximately 500,000 cells grown in a 6-well plate were washed twice with 1 x PBS then dissociated with 500 µl cell dissociation solution (Sigma C 5789) according to the supplier's instructions (Sigma). Cells were transferred to medium in a microcentrifuge tube and collected by centifugation at 2,500 rpm for 3 min. The supernatant was removed and cells resuspended in 1 ml 1 x PBS.

For fixation, cells were collected by centrifugation as above and suspended in 50 µl PBA (1 x PBS, 0.1 % w/v BSA fraction V (Trace) and 0.1 % w/v sodium azide) followed by the addition of 250 µl of 4 % w/v paraformaldehyde in 1 x PBS. and incubated at 4°C for 10 min. To permeabilize cells, cells were collected by centrifugation at 10,000 rpm for 30 sec, the supernatant removed and cells suspended in 50 µl 0.25 % w/v saponin (Sigma S 4521) in PBA and incubated at 4°C for 10 min. To block cells, cells were collected by centrifugation at 10,000 rpm for 30 sec, the supernatant removed and cells suspended in 50 µl PBA, 1 % v/v FBS and incubated at 4°C for 10 min.

To quantify HER-2 protein, fixed, permeabilized cells were probed with Anti-erbB2 monoclonal antibody at 1/100 dilution followed by Alexa Fluor 488 goat anti-mouse

IgG conjugate at 1/100 dilution. Cells were then analysed by FACS using a Becton Dickinson FACSCalibur and Cellquest software (Becton Dickinson). True background fluorescence values were established with unstained MDA-MB-468 cells and cells probed with an irrelevant primary antibody (MART-1, an IgG2b antibody (NeoMarkers)) and the Alexa Fluor 488 secondary antibody, both at 1/100 dilutions. Examples of FACS data are shown in Figure 74. Results of analyses of all cell lines are compiled in Table 10.

TABLE 10

Cell line	Mean Fluorescence	Geometric mean Fluorescence	Median Fluorescence
MDA-MB-468 control.1	5.07	4.72	4.78
MDA-MB-468 control.2	137.24	121.68	117.57
MDA-MB-468	1224.90	1086.47	1175.74
MDA-MB-468 1.1	1167.94	1056.17	1124.04
MDA-MB-468 1.4	781.72	664.67	673.17
MDA-MB-468 1.5	828.34	673.82	710.50
MDA-MB-468 1.6	925.16	807.09	850.53
MDA-MB-468 1.7	870.81	749.27	791.48
MDA-MB-468 1.8	1173.92	938.72	1124.04
MDA-MB-468 1.10	701.24	601.84	604.30
MDA-MB-468 1.11	1103.18	980.10	1064.99
MDA-MB-468 1.12	817.39	666.61	710.50
MDA-MB-468 2.5	966.72	862.76	905.80
MDA-MB-468 2.6	752.70	633.49	649.38
MDA-MB-468 2.7	842.00	677.15	716.92
,MDA-MB-468 2.8	986.05	792.13	881.68
MDA-MB-468 2.9	802.36	686.06	716.92
MDA-MB-468 2.10	1061.79	944.49	1009.04
MDA-MB-468 2.12	931.63	790.81	820.47
MDA-MB-468 2.13	894.47	792.46	827.88
MDA-MB-468 2.15	1052.87	946.79	1009.04
MDA-MB-468 3.1	1049.88	931.96	991.05
MDA-MB-468 3.2	897.00	802.43	842.91

MDA-MB-468 3.4	981.63	858.95	913.98
MDA-MB-468 3.5	1072.00	930.17	982.17
MDA-MB-468 3.7	1098.95	993.26	1036.63
MDA-MB-468 3.8	1133.86	1026.31	1074.61
MDA-MB-468 3.9	831.73	729.32	763.51
MDA-MB-468 3.12	1120.82	998.67	1064.99
MDA-MB-468 3.13	1039.41	963.71	1036.63
MDA-MB-468 4.5	770.93	681.01	697.83
MDA-MB-468 4.7	838.16	752.74	784.39
MDA-MB-468 4.8	860.76	769.51	813.12
MDA-MB-468 4.10	1016.21	904.69	947.46
MDA-MB-468 4.11	870.10	776.73	813.12
MDA-MB-468 4.12	986.93	857.20	913.98
MDA-MB-468 4.13	790.41	712.25	743.18
MDA-MB-468 4.14	942.36	842.34	873.79
MDA-MB-468 4.16	771.81	677.69	697.83

"MDA-MB-468 control.1" is MDA-MB-468 cells without staining — neither primary nor secondary antibody.

"MDA-MB-468 control.2" is MDA-MB-468 cells stained with irrelevant primary antibody MART-1 and the Alexa Fluor 488 secondary antibody.

All other cells, as described, were stained with Anti-erbB2 primary antibody and Alexa Fluor 488 secondary antibody.

These data show that MDA-MB-468 cells transformed with pCMV.HER2.BGI2.2REH have significantly reduced expression of HER-2 protein.

4. Southern analysis

Individual transgenic NIH/3T3 cell lines were analyzed by Southern blot to confirm integration of the transgene, according to the protocol set forth in Example 1.

5. Western blot analysis

Selected clones and control MDA-MB-468 cells were grown overnight to near-confluence on 100 mm TC plates (10^7 cells). Cells in plates were first washed with buffer containing phosphatase inhibitors (50mM Tris-HCl, pH 6.8, 1mM $Na_4P_2O_7$, 10mM NaF, 20μM Na_2MoO_4, 1mM Na_3VO_4), and then scraped from the plate in 600 μl of lysis buffer (50mM Tris-HCl, pH 6.8, 1mM $Na_4P_2O_7$, 10mM NaF, 20μM Na_2MoO_4, 1mM Na_3VO_4, 2% w/v SDS) which had been heated to 100°C. Suspensions were incubated in screw-capped tubes at 100°C for 15 min. Tubes with lysed cells were centrifuged at 13,000 rpm for 10 min and supernatant extracts were removed and stored at -20°C.

SDS-PAGE 10% v/v separating and 5% v/v stacking gels (0.75 mm) were prepared in a Protean apparatus (BioRad) using 29:1 acrylamide:bisacrylamide (Bio-Rad) and Tris-HCl buffers at pH 8.8 and 6.8, respectively. Volumes of 60 μl from extracts were combined with 20 μl of 4x loading buffer (50mM Tris-HCl, pH 6.8, 2% w/v SDS, 40% v/v glycerol, bromophenol blue and 400mM dithiothreitol added before use), heated to 100°C for 5 min, cooled then loaded into wells before the gel was run in the cold room at 120V until protein samples entered the separating gel, then at 240V. Separated proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham) using an electroblotter (Bio-Rad), according to the supplier's instructions.

Membranes were rinsed in TBST buffer (10mM Tris-HCl, pH 8.0, 150mM NaCl, 0.05% v/v Tween 20) then blocked in a dish in TBST with 5% w/v skim milk powder plus phosphatase inhibitors (1mM $Na_4P_2O_7$, 10mM NaF, 20μM Na_2MoO_4, 1mM Na_3VO_4). Membranes were incubated in a small volume in TBST with 2.5% w/v skim milk powder plus phosphatase inhibitors containing a mouse monoclonal

antibody against the ECD of HER-2 (Transduction Laboratories, NeoMarkers) diluted 1:4000. Membranes were washed three times for 10 min in TBST with 2.5% w/v skim milk powder plus phosphatase inhibitors. Membranes were incubated in a small volume in TBST with 2.5% w/v skim milk powder plus phosphatase inhibitors containing the horseradish peroxidase-conjugated secondary antibody diluted 1:1000. Membranes were washed three times for 10 min in TBST with 2.5% w/v skim milk powder plus phosphatase inhibitors.

The presence of HER-2 protein was detected using the ECL luminol-based system (Amersham), according to manufacturer's instructions. Several cell lines transformed with pCMV.HER2.BGI2.2REH showed greatly reduced or no detectable HER-2 protein.

EXAMPLE 13

Co-suppression of YB-1 and p53 in Murine Type B10.2 and Pam 212 cells in vitro

1. Culturing of cell lines

B10.2 cells (Immunex) derived from murine fibrosarcoma and Pam 212 cells (Auckland Medical School) derived from sponstaneously transformed murine epidermal keratinocytes were grown as adherent monolayers in cDMEM (DMEM with 0.77mM asparagine, 160μM penicillin G, 70μM dihydrostreptomycin sulfate) supplemented with 5% v/v FBS (B10.2) or 5% v/v equine serum (Pam 212), as described in Example 1, above.

2. Preparation of genetic constructs

(a) Interim plasmids

Plasmid TOPO.YB-1

To amplify a region of the mouse YB-1 gene, 25 ng of a plasmid clone containing

a mouse YB-1 cDNA (obtained from Genesis Research & Development Corporation, Auckland NZ) was used as a substrate for PCR amplification using the primers:-

Y1: AGA TCT GCA GCA GAC CGT AAC CAT TAT AGG [SEQ ID NO:25]

and

Y4: GGA TCC ACC TTT ATT AAC AGG TGC TTG CAG [SEQ ID NO:26].

The PCR amplification was performed using HotStarTaq DNA polymerase according to the supplier's protocol (Qiagen). PCR amplification conditions involved an initial activation step at 95°C for 15 min, followed by 35 amplification cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec, with a final elongation step at 72°C for 4 min.

The PCR-amplified region of YB-1 was column-purified (PCR purification column, Qiagen) and then cloned into pCR (registered trademark) 2.1-TOPO (Invitrogen) according to the supplier's instructions, to make plasmid TOPO.YB-1.

Plasmid TOPO.p53

To amplify a region of the mouse p53 gene, 25 ng of a plasmid clone containing a mouse p53 cDNA (obtained from Genesis Research & Development Corporation, Auckland, NZ) was used as a substrate for PCR amplification using the primers:-

P2: AGA TCT AGA TAT CCT GCC ATC ACC TCA CTG [SEQ ID NO:27]

and

P4: GGA TCC CAG GCC CCA CTT TCT TGA CCA TTG [SEQ ID

NO:28].

The PCR amplification was performed using HotStarTaq DNA polymerase according to the supplier's protocol (Qiagen). PCR amplification conditions involved an initial activation step at 95°C for 15 min, followed by 35 amplification cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec, with a final elongation step at 72°C for 4 min.

The PCR-amplified region of p53 was column-purified (PCR purification column, Qiagen) and then cloned into pCR (registered trademark) 2.1-TOPO (Invitrogen) according to the manufacturer's instructions, to make plasmid TOPO.p53.

Plasmid TOPO.YB1.p53

To create a construct fusing YB-1 and p53 cDNA sequences, the murine YB-1 sequence from TOPO.YB-1 was isolated as a *Bgl*II-to-*Bam*HI fragment and cloned into the *Bam*HI site of TOPO.p53. A clone in which the YB-1 insert was oriented in the same sense as the p53 sequence was selected and designated TOPO.YB1.p53.

(b) Test plasmids

Plasmid pCMV.YB1.BGI2.1BY

Plasmid pCMV.YB1.BGI2.1BY is capable of transcribing a region of the murine YB-1 gene as an inverted repeat or palindrome that is interrupted by the human β-globin intron 2 (BGI2) sequence therein. Plasmid pCMV.YB1.BGI2.1BY was constructed in successive steps: (i) the YB-1 sequence from plasmid TOPO.YB-1 was sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bgl*II-digested pCMV.BGI2 to make plasmid pCMV.YB1.BGI2, and (ii) the YB-1 sequence from plasmid TOPO.YB-1 was sub-cloned in the antisense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.YB1.BGI2 to make plasmid pCMV.YB1.BGI2.1BY.

Plasmid pCMV.YB1.p53.BGI2.35p.1BY

Plasmid pCMV.YB1.p53.BGI2.35p.1BY is capable of expressing fused regions of the murine YB-1 and p53 genes as an inverted repeat or palindrome that is interrupted by the human β-globin intron 2 (BGI2) sequence therein. Plasmid pCMV.YB1.p53.BGI2.35p.1BY was constructed in successive steps: (i) the YB-1.p53 fusion sequence from plasmid TOPO.YB1.p53 was sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bgl*II-digested pCMV.BGI2 to make plasmid pCMV.YB1.p53.BGI2, and (ii) the YB-1.p53 fusion sequence from plasmid TOPO.YB1.p53 was sub-cloned in the antisense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.YB1.p53.BGI2 to make plasmid pCMV.YB1.p53.BGI2.35p.1BY.

3. Detection of co-suppression phenotypes

(a) Post-transcriptional gene silencing of YB-1 by insertion of a region of the YB-1 gene into murine fibrosarcoma B10.2 cells and murine epidermal keratinocyte Pam 212 cells

YB-1 (Y-box DNA/RNA-binding factor 1) is a transcription factor that binds, *inter alia,* to the promoter region of the p53 gene and in so doing represses its expression. In cancer cells that express normal p53 protein at normal levels (some 50% of all human cancers), the expression of p53 is under the control of YB-1, such that diminution of YB-1 expression results in increased levels of p53 protein and consequent apoptosis. The murine cell lines B10.2 and Pam 212 are two such tumorigenic cell lines with normal p53 expression. The expected phenotype for co-suppression of YB-1 in these two cell lines is apoptosis.

Transformations with pCMV.YB1.BGI2.1BY were performed in 6-well tissue culture vessels. Individual wells were seeded with 3.5 x 10^4 cells (B10.2 or Pam 212) in 3 ml of cDMEM, 5% v/v FBS (B10.2) or equine serum (Pam 212) and incubated at 37°C in 5% v/v CO_2 for 24 hr prior to transfection.

The two mixes used to prepare transfection medium were:

Mix A: 1.5 μl of LIPOFECTAMINE 2000 (trademark) Reagent in 100 μl of OPTI-MEM I (registered trademark), incubated at room temperature for 5 min;

Mix B: 1 μl (400 ng) of pCMV.YB1.BGI2.1BY DNA in 100 μl of OPTI-MEM I (registered trademark) medium.

After preliminary incubation, Mix A was added to Mix B and the mixture incubated at room temperature for a further 20 min.

Medium overlaying each cell culture was replaced with 800 μl of fresh medium and 200 μl of transfection mix added. Cells were incubated at 37°C in 5% v/v CO_2 for 72 hr.

Duplicate cultures of both cell types (B10.2 and Pam 212) were transfected.

Cells were suspended with trypsin, centrifuged and resuspended in PBS according to the protocol described in Example 1.

Live and dead cell numbers were determined by trypan blue staining (0.2%) and counting in quadruplicate on a haemocytometer slide. Results are presented in Figure 75.

(b) Post-transcriptional gene silencing of YB-1 and p53 by co-insertion of regions of the YB-1 and p53 genes into murine fibrosarcoma B10.2 cells and murine epidermal keratinocyte Pam 212 cells

The data presented in Figure 75 show that cell death is increased in B10.2 and Pam 212 cells following insertion of a YB-1 construct designed to induce co-suppression of YB-1, consistent with induction of co-suppression.

Simultaneous co-suppression of p53, which is responsible for initiating the

apoptotic response in these cells, would be expected to eliminate excess cell death by apoptosis.

Transformations with pCMV.YB1.p53.BGI2.35p.1BY were performed in 6-well tissue culture vessels. Individual wells were seeded with 3.5 x 10^4 cells (B10.2 or Pam 212) in 3 ml of cDMEM, 5% v/v FBS (B10.2) or equine serum (Pam 212) and incubated at 37°C in 5% v/v CO_2 for 24 hr prior to transfection.

The two mixes used to prepare transfection medium were:-

Mix A: 1.5 µl of LIPOFECTAMINE 2000 (trademark) Reagent in 100 µl of OPTI-MEM I (registered trademark) medium, incubated at room temperature for 5 min;

Mix B: 1 µl (400 ng) of pCMV.YB1.p53.BGI2.35p.1BY DNA in 100 µl of OPTI-MEM I (registered trademark) medium.

After preliminary incubation, Mix A was added to Mix B and the mixture incubated at room temperature for a further 20 min.

Medium overlaying each cell culture was replaced with 800 µl of fresh medium and 200 µl of transfection mix added. Cells were incubated at 37°C in 5% v/v CO_2 for 72 hr.

Cells were suspended with trypsin, centrifuged and resuspended in PBS according to the protocol described in Example 1.

Live and dead cell numbers were determined by trypan blue staining (0.2%) and counting in quadruplicate on a haemocytometer slide. Results are presented in Figure 75.

(c) Control: Insertion of EGFP into murine fibrosarcoma B10.2 cells and murine epidermal keratinocyte Pam 212 cells

Transformations with pCMV.EGFP were performed in 6-well tissue culture vessels. Individual wells were seeded with 3.5×10^4 cells (B10.2 or Pam 212) in 3 ml of cDMEM, 5% v/v FBS (B10.2) or equine serum (Pam 212) and incubated at 37°C in 5% v/v CO_2 for 24 hr prior to transfection.

The two mixes used to prepare transfection medium were:-

Mix A: 1.5 µl of LIPOFECTAMINE 2000 (trademark) Reagent in 100 µl of OPTI-MEM I (registered trademark) medium, incubated at room temperature for 5 min;

Mix B: 1 µl (400 ng) of pCMV.EGFP DNA in 100 µl of OPTI-MEM I (registered trademark) medium.

After preliminary incubation, Mix A was added to Mix B and the mixture incubated at room temperature for a further 20 min.

Medium overlaying each cell culture was replaced with 800 µl of fresh medium and 200 µl of transfection mix added. Cells were incubated at 37°C in 5% v/v CO_2 for 72 hr.

Cells were suspended with trypsin, centrifuged and resuspended in PBS according to the protocol described in Example 1.

Live and dead cell numbers were determined by trypan blue staining (0.2%) and counting in quadruplicate on a haemocytometer slide. Results are presented in Figure 75.

(d) Control: Attenuation of YB-1 phenotype by insertion of a decoy Y-box oligonucleotide into murine fibrosarcoma B10.2 cells and murine epidermal

keratinocyte Pam 212 cells

The role of YB-1in repressing p53-initiated apoptosis in B10.2 and Pam 212 cells has been demonstrated by relieving the repression in two ways: (i) transfection with YB-1 antisense oligonucleotides; (ii) transfection with a decoy oligonucleotide that corresponds to the YB-1 cis element derived from the *fas* silencer region (-1035 to -1008 of the 5'-flanking sequence of the human *fas* gene). The latter was used as a positive control in the present example.

The double-stranded oligonucleotides used were:

YB1 decoy: GAA CCT GAA TTT GGA TGC AGT TCC AGA C [SEQ ID NO:29]

CTT GGA CTT AAA CCT ACG TCA AGG TCT G

YB1 control: GCG GAT AAC AAT TTC ACA CAG G [SEQ ID NO:30]

CGC CTA TTG TTA AAG TGT GTC C

Transformations with YB1 decoy and a control (non-specific) oligonucleotide were performed in 24 well tissue culture vessels. Individual wells were seeded with 3.5 x 10^4 cells (B10.2 or Pam 212) in 3 ml of cDMEM, 5% v/v FBS (B10.2) or equine serum (Pam 212) and incubated at 37°C, 5% v/v CO_2 for 24 hr prior to transfection.

The two mixes used to prepare transfection medium were:-

Mix A: 1.5 μl of Lipofectin (trademark) Reagent (Life Technologies) in 100 μl of OPTI-MEM I (registered trademark) medium, incubated at room temperature for 30 min;

Mix B: 0.4 μl (40 pmol) of oligonucleotide (YB1 decoy or control) in 100 μl of OPTI-MEM I (registered trademark) medium.

After preliminary incubation, Mix A was added to Mix B and the mixture incubated at room temperature for a further 15 min.

A no-oligonucleotide (Lipofectin (trademark) only) control was also prepared.

Cells were washed in serum-free medium (OPTI-MEM I (registered trademark)) and transfection mix added. Cells were incubated at 37°C in 5% v/v CO_2 for 4 hr, after which medium was replaced with 1 ml of cDMEM containing 5% v/v serum and incubation continued overnight (18 hr).

Cells were suspended with trypsin, centrifuged and resuspended in PBS according to the protocol described in Example 1.

Live and dead cell numbers were determined by trypan blue staining (0.2%) and counting in quadruplicate on a haemocytometer slide. Results are presented in Figure 75.

TABLE 2

Plasmid construct	No of plants tested	Percentage of plants showing specified phenotype		
		Susceptible	Immune	Resistant
pART27.PVY	19	16	1	2
pART27.PVYx2	13	5	4	4
pART27.PVYx3	21	2	5	14
pART27.PVYx4	21	5	7	9
pART27.35S.PVY.SCBC.0	25	8	0	17
pART27.35S.O.SCBV.PVY	22	8	0	14
pART27.35S.O.SCBV.YVP	18	14	0	4
pART27.35S.PVY.SCBV.YVP	17	3	8	6
pART27.PVY.LNYV.PVY	26	18	2	6
pART27.PVY.LNYV.YVP	20	6	10	4

pART27.PVY.LNYV.YVPΔ	18	7	11	0

Post-transcriptional effect

The experiment was carried out to demonstrate the effect that the silencing was at least, in part, post-transcriptional.

Referring to Figure 76, there is shown a Northern blot as explained in the description of Figure 76 set out above. This blot shows that clone #18 may be regarded as a "positive control", in that there was no significant silencing. However, clones #3 and #9 do show silencing as can be seen from the absence of a band in their respective lanes in the Northern blot of Figure 76.

Figure 77 graphs the results of Real-Time RT-PCR analysis of these cell lines, as explained in the description of Figure 77 set out above. While it would be apparent to one skilled in the art the method used to generate these graphs, the method is set out in more detail in US Provisional Patent application serial no:60/316,308.

The results of these graphs are tabulated for reference purposes in the table set out in Figure 78. Referring to Figure 78, it can be seen that the results for clone #18 show that the mRNA level for EGFP is set as the standard (1.000) relative to which other mRNA levels are measured. It can be seen that there is also significant mRNA present for GAPD (little under half the amount for EGFP). Under the heading "Transcription", it can be seen that there is a measurable rate of mRNA transcription for EGFP and a lower rate of transcription measurable for GAPD. The results for clone #3 are shown on the second line. Consistent with the results of Figure 76, there is no mRNA for EGFP although there is still mRNA for GAPD. Further, the rate transcription for EGFP is negligible but significant for GAPD. This demonstrates that EGFP is silenced at the transcription point, with GAPD expression showing that other genes are not silenced. Finally, the last line shows the results for clone #9. Again, there is no detectable mRNA for EGFP,

which is again consistent with the EGFP having been silenced as shown in Figure 76. The mRNA level for the GAPD again shows that other genes are being transcribed into mRNA. However, the significance of these results is that there is a significant level of EGFP transcription measured (as if course there is for GAPD as well). Thus, in clone #9, EGFP is being transcribed but the lack of mRNA for EGFP shows that the silencing must be occurring post-transcriptionally.

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